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	PAT. NO.	Title
1	6,596,768	Unsaturated lipid-enriched feedstock for ruminants
2	6,596,733	Use of nitric oxide scavengers to treat side effects caused by therapeutic administration of sources of nitric oxide
3	6,596,332	Gelatinized cereal product containing oligosaccharide
4	6,591,133	Apparatus and methods for fluid delivery using electroactive needles and implantable electrochemical delivery devices
5	6,589,990	Methods and compositions for misoprostol compound treatment of erectile dysfunction
6	6,585,764	Stent with therapeutically active dosage of rapamycin coated thereon
7	6,584,472	Method, system and article for creating and managing proprietary product data
8	6,572,915	Process for enriching foods and beverages
9	6,572,542	System and method for monitoring and controlling the glycemic state of a patient
10	6,572,040	Coal grinding, cleaning and drying processor
11	6,569,865	Spiro 1-azabicyclo[2.2.2]octane-3,2'(3'h)-furo[2,3-b]pyridine
12	6,569,147	Systems and methods of use for delivering beneficial agents for revascularizing stenotic bypass grafts and other occluded blood vessels and for other purposes
13	6,569,099	Ultrasonic method and device for wound treatment
14	6,562,807	Glucagon antagonists/inverse agonists
15	6,562,235	Enhanced anaerobic treatment zones in groundwater
16	6,562,065	Expandable medical device with beneficial agent delivery mechanism
17	6,561,437	Water and fertilizer dispenser
18	6,558,550	Process for treating septage

19 6,555,001 Accelerated remediation using tree crops
20 6,546,106 Acoustic device
21 6,544,499 Topical compositions comprising protected functional thiols
22 6,544,252 Osmotic delivery system having space efficient piston
23 6,539,250 Programmable transdermal therapeutic apparatus
24 6,533,803 Wound treatment method and device with combination of ultrasound and laser energy
25 6,532,710 Solid monolithic concrete insulated wall system
26 6,531,272 Color photographic element containing a fragmentable electron donor for improved photographic response
27 6,524,848 Recirculating composting system
28 6,524,800 Exploiting genomics in the search for new drugs
29 6,524,305 Osmotic delivery system flow modulator apparatus and method
30 6,522,760 Active acoustic devices
31 6,520,982 Localized liquid therapy and thermotherapy device
32 6,516,749 Apparatus for the delivery to an animal of a beneficial agent
33 6,511,969 Method for reducing coronary artery reactivity
34 6,508,808 Valve for osmotic devices
35 6,500,134 Method for treating circulatory disorders with acoustic waves
36 6,490,826 Methods of utilizing a finely divided mineral composite
37 6,490,565 Environmental certification system and method
38 6,489,322 Amidine derivatives as inhibitors of nitric oxide synthase
39 6,488,997 Degradable composite material, its disposable products and processing method thereof
40 6,488,940 Use of 17-.alpha.-estradiol for the treatment of aged or sundamaged skin and/or skin atrophy
41 6,488,652 Safety valve assembly for implantable benefical agent infusion device
42 6,486,205 Mixture of primary fatty acids obtained from sugar cane wax
43 6,485,765 Lactational performance of dairy cattle
44 6,484,204 System and method for allocating requests for objects and managing replicas of objects on a network
45 6,482,234 Prosthetic spinal disc
46 6,482,092 Image-display game system and information storage medium used therefor
47 6,479,024 Process for removing impurities from bauxite
48 6,478,754 Ultrasonic method and device for wound treatment
49 6,478,109 Laminated composite panel-form loudspeaker
50 6,474,338 Device and method for simultaneously delivering beneficial agents to both cervical and vaginal lumen sides of a vagina

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Refine Search isd/19900101->20030728 and aclm/beneficial and cc

	PAT. NO.	Title
1	6,544,499	Topical compositions comprising protected functional thiols
2	6,544,252	Osmotic delivery system having space efficient piston
3	6,524,305	Osmotic delivery system flow modulator apparatus and method
4	6,508,808	Valve for osmotic devices
5	6,488,940	Use of 17-.alpha.-estradiol for the treatment of aged or sundamaged skin and/or skin atrophy
6	6,486,205	Mixture of primary fatty acids obtained from sugar cane wax
7	6,468,961	Gel composition and methods
8	6,468,779	Aqueous extract of legumes for growing agronomically beneficial microbes
9	6,468,525	Probiotic formulation
10	6,451,337	Chitosan-based nitric oxide donor compositions
11	6,391,324	Cosmetic skin care compositions containing pulegone
12	6,383,506	Compositions containing colorants and microorganisms for treating natural bodies of water
13	6,365,185	Self-destructing, controlled release peroral drug delivery system
14	6,337,318	Sustained GnRH peptide-release formulation
15	6,333,052	Medicaments for beneficial insects and method
16	6,331,311	Injectable depot gel composition and method of preparing the composition
17	6,284,537	Immortalized human corneal epithelial cell line
18	6,283,953	Osmotic drug delivery monitoring system and method
19	6,277,365	Ophthalmic composition including a cationic glycoside and an anionic therapeutic agent
20	6,270,787	Osmotic delivery system with membrane plug retention mechanism
21	6,267,984	Skin permeation enhancer compositions comprising a monoglyceride and ethyl palmitate

22 6,261,594 [T] Chitosan-based nitric oxide donor compositions
23 6,251,432 [T] Sustained release dosage form unit having latex coating and method of making the same
24 6,241,983 [T] Bacteria-and fiber-containing composition for human gastrointestinal health
25 6,231,894 [T] Treatments based on discovery that nitric oxide synthase is a paraquat diaphorase
26 6,221,893 [T] Administration of histamine for therapeutic purposes
27 6,217,906 [T] Self adjustable exit port
28 6,211,152 [T] Formulations for peptide release
29 6,203,797 [T] Dietary supplement and method for use as a probiotic, for alleviating the symptons associated with irritable bowel syndrome
30 6,197,342 [T] Use of biologically active glass as a drug delivery system
31 6,197,334 [T] Lozenge and method of making
32 6,180,129 [T] Polyurethane-containing delivery systems
33 6,180,099 [T] Method of using immunoglobulin and fiber-containing compositions for human health
34 6,162,471 [T] Thickened hydrolyte isotonic beverage
35 6,156,354 [T] Hyper-absorption of vitamin E dispersed in milks
36 6,153,221 [T] Use of biogically active glass as a drug delivery system
37 6,153,213 [T] Means for supplying a beneficial substance to an animal
38 6,132,420 [T] Osmotic delivery system and method for enhancing start-up and performance of osmotic delivery systems
39 6,130,200 [T] Gel composition and methods
40 6,113,938 [T] Beneficial agent delivery system with membrane plug and method for controlling delivery of beneficial agents
41 6,110,480 [T] Method for preparing an environmentally compatable porous material comprising beneficial nematodes and the biotic preparations produced therefrom
42 6,087,334 [T] Anti-diabetic peptides
43 6,083,534 [T] Pharmaceutical compositions for controlled release of soluble receptors
44 6,033,713 [T] Thickened hydrolyte isotonic beverage
45 6,001,279 [T] Solidified water soluble wood preservative and method of making the same
46 5,997,527 [T] Self adjustable exit port
47 5,993,221 [T] Dietary formulation comprising arachidonic acid and methods of use
48 5,985,861 [T] Progesterone for treating or reducing ischemia
49 5,985,314 [T] Bolus for supplying biologically beneficial substances to ruminant animals
50 5,980,509 [T] Osmotic system for delivery of fluid-sensitive somatotropins to bovine animals

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US006486205B2

(12) **United States Patent**
Gonzáles Bravo et al.

(10) **Patent No.: US 6,486,205 B2**
(45) **Date of Patent: *Nov. 26, 2002**

(54) **MIXTURE OF PRIMARY FATTY ACIDS OBTAINED FROM SUGAR CANE WAX**

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(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 114 days.

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Apr. 2, 1997 (CU) 35/97

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(58) **Field of Search** 514/553, 557, 514/558, 560, 822, 925, 960; 424/464; 426/425, 429, 431, 472, 478, 655

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Primary Examiner—Allen J. Robinson
Assistant Examiner—Frank Choi
(74) *Attorney, Agent, or Firm*—Lorusso & Loud

(57) **ABSTRACT**

A new natural mixture of primary fatty acids of high molecular weight ranging from 24 to 38 carbon atoms, especially those ranging between 26 and 36 carbon atoms and more especially those of straight chain of 26, 28, 29, 30, 31, 32, 33, 34, 35 and 36 carbon atoms. This mixture has a relative composition of each fatty acid that is highly reproducible batch to batch and it is extracted from sugar cane (*Saccharum officinarum*, L.) wax. This mixture of fatty acids has specific pharmacological properties that supports its use as an active component of pharmaceutical formulations used as hypocholesterolemic and against hypercholesterolaemia type II, as antiplatelet, anti-thrombotic and anti-ischemic. This mixture of primary fatty acids is also effective in the inhibition of the development of gastric ulcers induced by different agents.

19 Claims, No Drawings

MIXTURE OF PRIMARY FATTY ACIDS OBTAINED FROM SUGAR CANE WAX

This application is a 371 of PCT/1B98/00780 filed Apr. 1, 1998.

This invention relates to a new mixture of primary fatty acids with straight chains of 26, 28, 29, 30, 31, 32, 33, 34, 35 and 36 carbon atoms. This mixture shows a relative composition of the fatty acids that is highly reproducible from batch to batch and it is extracted from sugar cane (Saccharum officinarum L.) wax, and can be used for the treatment of type II hypercholesterolemia, as antiplatelet agent, as anti-thrombotic, as anti-ischemic agent as well as protective and/or curative agent against gastric ulcers.

There are other mixtures of fatty acids that has reported biological properties, such is the case of the one reported in U.S. Pat. No. 5,284,873 in which is claimed a pharmaceutical composition for combatting prostate affections, in which the active principle is a fraction of fatty acids obtained from the fruits of Sabal Serrulata, with oleic acid as the main one, followed by lauric and palmitic acids, being the one with the greater number of carbon atom eicosenoic. In the U.S. Pat. No. 5,502,045 is claimed a method of reducing the cholesterol levels in serum using an ester formed by a-sitostanol and a 2 fatty acid from 2 to 22 carbon atoms. Also, claims a method of formation of such esters, being the effective daily dose of 0.2 to 20 g of the ester. Although, in the U.S. Pat. No. 5,444,054 is reported a method of treating ulcerative colitis in which one of the fractions used is an oil that contains certain fatty acids from 18 to 22 carbon atoms, but unsaturated. Not close to this patent application are the U.S. Pat. Nos. 4,505,933 and 4,687,783 in which are claimed mixtures of fatty aldehydes derived from fatty acids, for the treatment of patients with multiple sclerosis as well as neurological and dermatological diseases, the inventors claims a daily dose of 100–400 mg/kg of body weight of the patients.

In the last decade, numerous patent have appeared which report the omega-3-poly-unsaturated fatty acids have an effect on serum cholesterol and blood platelet aggregation, such is the case of U.S. Pat. No. 4,526,902 where it is claimed a pharmaceutical composition for the treatment of thrombo-embolic conditions in which the active principle are unsaturated eicosapentaenoic and docohexacnoic fatty acids together with linoleic acid, linolenic acid and its derivatives. Also, in the U.S. Pat. No. 5,502,077 is claimed a composition of fatty acids for the treatment or prophylaxis of multiple risk factors for cardiovascular diseases, where the 80% in weight corresponds with omega-3 fatty acids, its salts or derivatives thereof, being the main ones eicosapentaenoic and docohexacnoic acids. In EPO patent 0 422 490 A2 it is claimed a pharmaceutical composition for inhibiting the absorption of cholesterol, containing triglycerides formed, preferably, by a mixture of saturated fatty acids from 20 to 24 carbon atoms, but they should be administered in a daily dose of 2 to 10 g. Also, in the Japanese patents 55092316 A and 56115736 A. of Tokiwa Shizeru et al, is reported the demonstration as well as the isolation and purification of an agent for diminishing cholesterol composed by a mixture of highly unsaturated fatty acids, especially eicosatrienoic and docosatertraenoicacids. In other Japanese patents (publication # 1290625 A, 02053724 A, 02243622 A and 04169524 A) claimed different pharmaceutical formulations, such as an improver of cerebral function or for the treatment of degenerative disease, or for lowering cholesterol in blood and for having Serum lipid-improving activity in which are presents as active principle, mixtures of fatty acids, especially eicosapentaenoic and docosahexacnoic acids, in a daily dose that varies from 500 mg/kg of body weight to 0.5–30 g.

As it has been shown, these patents claims mixture of fatty acid with specific biological properties, some of them closely related with the ones claimed in the present patent application. But, the composition of these mixtures significantly differs from the one claimed in these patent application and, also, the proposed daily dose to be used in these treatments in higher than the one claimed in the present patent application.

The present invention is related mainly with the pharmaceutical industry, particularly with the development of pharmaceutical formulations with specific properties, because them could be used as hypocholesterolaemic and hypolypoproteinaemic, anti-platelet anti-thrombotic, anti-ischemic drugs as well as in the prevention of gastric and duodenal ulcers induced by different agents.

These formulations contains, as active principle, a natural mixture of primary fatty acids of high molecular weight ranging from 24 to 38 carbon atoms, especially those ranging from 26 to 36 carbon atoms and more especially those of straight chain of 26, 28,29,30,31,32,33,34,35 and 36 carbon atoms, obtained from sugar cane wax (named MFASCW).

Drugs with specific pharmacological properties, based in the uses of primary fatty acids of high molecular weight (from 26 to 36 carbon atoms) obtained from, vegetable or animal waxes as active principles has not been reported previously, but it is known that primary fatty alcohols of high molecular weight obtained from sugar cave wax shows the same pharmacological properties. In EPO patent 0 488 928 A2 is claimed a mixture of fatty alcohols of high molecular weight for the treatment of hypercholesterolaemia and hyperlypoproteinaemia, type II that., also, in patent WO 94/07830 is claimed the use of the same mixture as anti-platelet agent, anti-ischemic, anti-thrombotic as well as in the prevention of gastric and duodenal ulcers induced by different agents.

The procedure for the obtention of this mixture of primary fatty acids of high molecular weight in the present invention is based in an homogeneous saponification of sugar cane wax with concentrated solutions of alkaline and earth-alkaline hydroxides, especially those of low molecular weight and more especially those of sodium, potassium and calcium. The concentration of the alkaline solution must be such that the ratio in weight of the corresponding hydroxide with that of the wax to be processed must be over 5% on, especially from 8 to 25% and more specifically from 15 to 25%. Saponification process lasts for a period over 30 min and more especially between 1 to 5 hours. The solid, obtained in this step, is processed using a conventional solid-liquid extractor, where the M.F.A.S.C.W., in salt form, is isolated from the rest of the components by extracting these components using the adequate organic solvent choose among ketones from 3 to 8 carbon atoms, alcohols from 1 to 5 carbon atoms, hydrocarbons from 5 to 8 carbon atoms, haloforms as well as aromatic compounds including mixtures of them. Some preferred solvents used in the present invention are the following: acetone, methyl-ethyl ketone, pentanone, hexanone, terbutanol, ethanol, methanol, 2-propanol, butanol, hexane, pentane, isopentane, ciclohexane, heptane, chloroform, 1,2 dichloroethane, dichloromethane, trichloroethane, trichloromethane, 1,2,3 trichloropropane, benzene, toluene, phenol, p-methyl toluene and others.

The mixture of primary fatty acids of high molecular weight in salt form is purified by succesive recristalizations

in an adequate organic solvents or in aqueous solutions, choosen among ketones from 3 to 8 carbon atoms, alcohols from 1 to 5 carbon atoms, hydrocarbons from 5 to 8 carbon atoms, as well as aromatic compounds, including mixtures of them. Some preferred solvents used in this step of the process acetone, methyl-ethyl ketone, pentanone, hexanone, terbutanol, ethanol, methanol, 2-propanol, butanol, hexane, pentane, heptane, octane, ciclohexane, benzene, toluene, phenol, p-methyl toluene and water between others.

An step in the purification of this mixture of primary fatty acids of high molecular weight consist in refluxing the components in an adequate organic solvent, choose among ketones from 3 to 8 carbon atoms, alcohols from 1 to 5 carbon atoms, hydrocarbons from 5 to 8 carbon atoms as well as aromatic compounds including mixtures of them and, immediately, hot filtering the fatty acids salts.

In the final step of this process the free fatty acids are regenerated using an acid solution, that could be prepared using mineral acids and/or organic acids to be choose among hydrochloric acid, sulphuric acid, nitric acid, perchloric acid, acetic acid and oxalic acid among others. The yield of fatty acid is between 10 to 40%, while the purity of the M.F.A.S.C.W. is in the general range from 85 to 100%, more especially between 90 to 99%, determined using gas chromatography and/or volumetric chemical analysis.

The M.F.A.S.C.W. obtained in the present invention is a mixture of primary fatty acids of high molecular weight ranging from 26 to 36 carbon atoms, more especially the one of 26, 28, 29, 30, 31, 32, 33, 34, 35 and 36 carbon atoms. In Table I is reported the qualitative and quantitative composition of this M.F.A.S.C.W.

TABLE I

Qualitative and quantitative composition of the M.F.A.S.C.W.

Component	Percent in the mixture (%)
1-hexacosanoic	0.3–1.5
1-octacosanoic	25.0–50.0
1-nonacosanoic	1.0–3.0
1-triacontanoic	15.0–30.0
1-hentriacontanoic	0.8–3.0
1-dotriacontanoic	10.0–22.0
1-tritriacontanoic	1.0–3.0
1-tetratriacontanoic	10.0–22.0
1-pentatriacontanoic	0.5–1.5
1-hexatriacontanoic	2.0–9.0

The daily dose of M.F.A.S.C.W. to be used for the treatment of the different diseases has been established between 1 to 100 mg per day and the most adequate route of administration is oral solid dosage-form such as tablets, gragees or capsules. Also, this drug could be administered orally or parenterally or topically considering the uses claimed in the present invention.

The pharmaceutical formulation, used in the oral route contains as active principle from 0.5 to 25.0% in weight of M.F.A.S.C.W. This dose is obtained by mixing M.F.A.S.C.W. with different excipients such as disintegrators, agglutinants, lubricants, sliders or just fillers.

One of the objects of the present invention is to isolate and purify the natural mixture of primary fatty acis with high molecular weight ranging from 26 to 36 carbon atoms from sugar cane wax, especially the mixture of straight chain fatty acids of 26, 28, 29, 30, 31, 32, 33, 34, 35 and 36 carbon atoms.

Other of the objects of this invention is to use this natural mixture of fatty acids, in relatively low doses, as a component of pharmaceutical formulations used as hypocholesterolemic and hypolipidaemic drugs. Also, one of the objects of the present invention is the development of pharmaceutical formulations that contains M.F.A.S.C.W. as active principle in order to be used as anti-ischemic and anti-thrombotic drugs, administered orally, topically or parenterally.

Finally, in a whole picture of M.F.A.S.C.W. profile, and proposed as active principle for pharmaceutical formulations, this mixture is innocuos, and very well tolerated, representing an important advantage. Thus, results obtained in toxicological assays carried out in rodents report absence of toxicity related with the M.F.A.S.C.W.. No side effects have been detected in subjects treated with the product which is object of the present invention. The object of the current invention shall be described in detail in the following pages. References will be made to examples of accomplishments that are not limited to the scope of the said invention.

EXAMPLE 1

One thousand (1000) g of refined sugar cane wax were taken and melted at 100–110° C. and 200 g of potassium hydroxide are added dissolved in 150 mL of water. The saponification process is maintained for 30 min with stirring periodically. The fatty acid is extracted from the solid obtained in the process using acetone in a solid-liquid extraction system. The obtained residue is cooled to room temperature, recrystallized in heptane and, later on, is refluxed with methanol for 2 h, with a final hot filtration. M.F.A.S.C.W. is regenerated by treatment with concentrate sulphuric acid. 250 g of this M.F.A.S.C.W. were obtained with a purity amounting to 94.0%.

EXAMPLE 2

Ten (10) kg of refined sugar cane wax were taken and melted at 100–110° C. to which 2 kg of sodium hydroxide dissolved in 1.5 L of water was added. The hydrolisis process is maintained for a period of two hours, stirring periodically. The solid obtainec in the process is extracted with ethanol in a conventional solid-liquid extraction system. The extract obtained was cooled to room temperature, the solid obtained was recrystallized in acetone and, later on, is refluxed for two hours in pentane, with a final hot filtration. M.F.A.S.C.W. was obtained by treatment of the solid with concentrate nitric acid and 2.45 kg of M.F.A.S.C.W. were aobtained with a purity amounting 95.0%.

EXAMPLE 3

Ten (10) kg of raw sugar cane wax were taken and melted adding 2.5 kg of calcium hydroxide dissolved in water. The saponification process was maintained for 30 min, with periodically stirring. The solid obtained in the process was extracted with acetone in a solid-liquid extraction system. The residue obtained in this step of the process was cooled to room temperature, recrystallized in benzene and, later on, refluxed for one hour in toluene for a final hot filtration. M.F.A.S.C.W. was obtained by treatment of the solid with concentrate sulphuric acid. 2.3 kg of M.F.A.S.C.W. were obtained with a purity amounting 93.5%.

EXAMPLE 4

Fifty (50) kg of refined sugar cane wax were taken and melted and enough quantity of potassium hydroxide dissolved in water were added. The saponification process was maintained for 30 min stirring periodically. The solid

obtained in the process was extracted with chloroform in a solid-liquid extraction system. The residue obtained in this step of the process was cooled at room temperature and recrystallized in methyl ethyl ketone. Later on, it was refluxed for 3 hours in methanol for a final hot filtration. M.F.A.S.C.W. was obtained by treatment of the solid with concentrate hydrochloric acid. 13.6 kg of M.F.A.S.C.W. were obtained with a purity amounting 96.0%

EXAMPLE 5

One thousand (1000) g of refined sugar cane wax were taken to be melted at 100–110° C. adding 250 g of potassium hydroxide dissolved in 250 mL of water and the wax was saponified for 1 hour with periodically stirring. The solid obtained in the process was extracted with n-hexane in a solid-liquid extraction system for 12 h. The residue obtained in this step of the process was cooled at room temperature, recrystallized in ethanol and, later on, refluxed in acetone for 30 min with a final hot filtration. M.F.A.S.C.W. was regenerated by treatment with an aqueous solution of sulphuric acid. Two hundreds (250) g of M.F.A.S.C.W. were obtained with a purity amounting 96.7%.

EXAMPLE 6

Two different pharmaceutical formulations, tablets type, using M.F.A.S.C.W. as active principle, are developed. The composition of these pharmaceutical formulations are presented in Table II. These formulations were developed considering the physical, chemical and chemical-physical characteristics of the active principle.

TABLE II

Pharmaceutical formulations tablets type using M.F.A.S.C.W. as active principle

Component	Formulation 1	Formulation 2
	(%)	
M.F.A.S.C.W.	5.0	15.0
Lactose	56.5	55.0
Corn starch	15.0	10.0
Gelatin	2.5	2.0
Sodium croscarmellose	5.0	4.0
Talc	2.0	2.0
Magnesium stearate	1.5	1.0
Mycrocrystalline cellulose	7.5	7.0

EXAMPLE 7

The object of this work is to evaluate the effect of M.F.A.S.C.W. on the platelet aggregation induced in rats. A group of male Sprague Dawley weighing 250 to 300 g were adapted to laboratory conditions (25+/31 2° C., ligth/darkness cycles of 12 h) with free access to water and food for 15 days. M.F.A.S.C.W. was prepared as a suspension in an Acacia gum/water vehicle (10 mg/mL) and orally administered by gastric gavage. Animals were randomly distributed among three experimental groups: 1) controls, animals receiving vehicle in an equivalent volume; 2) animals receiving M.F.A.S.C.W. 20 mg/kg and 3) animals receiving M.F.A.S.C.W. 200 mg/kg. All animals were deprived of food for 12 h prior to the administration of the drug. To conduct the assay, rats were anaesthetized with sodium pentobarbital and 30 min after the administration of the drug, the abdomens were opened and blood was drawn from the vein cava mixed with sodium citrate (1 voluime of citrate per 9 of blood). Platelet-rich plasma (PRP) was obtained by blood centrifugation for 10 min at 160 g. Platelet-poor plasma (PPP) was obtained by centrifugation of plama aliquots for 5 min at 2500 g. Platelet aggregation was determined according to the Born method, using an Elvi -840 aggregometer at 37° C. and stirring (1000 rpm). Platelet aggregation levels were measured after calibrating the equipment at 0% light transmission for PRP and at 100% for PPP. ADP (Sigma) was used for inducing platelet aggregation.

The statistical comparison of results between treatment and control groups was carried out using the nonparametric Mann-Whitney U Test.

The M.F.A.S.C.W. (20 and 200 mg/kg) administered in unique doses showed a significant inhibition of platelet aggregation induced by ADP in rats, as is shown in Table III. These results corroborate the M.F.A.S.C.W. acts as an antiplatelet drug.

TABLE III

Effect of M.F.A.S.C.W. on platelet aggregation in rats

Treatment	Dose (mg)kg)	n	5 mM ADP % of platelet aggregation (values are the mean ± SD)
Control		12	39.5 ± 12.6
M.F.A.S.C.W.	20	6	24.2 ± 7.5*
M.F.A.S.C.W.	200 mg/kg	5	19.8 ± 4.3**

* $p < 0.05$; ** $p < 0.01$ Comparison with controls. (Mann-Whitney U test).

EXAMPLE 8

In this study were investigated the effects of M.F.A.S.C.W. on veinous thrombosis experimentally induced in rats and its posible effects on the bleeding time. Male Sprague Dawley rats weighing 300 to 350 g were adapted to laboratory conditions (25±2° C., ligth/darkness cycles of 12 h) with free access to water and food for 7 days. M.F.A.S.C.W. was orally administered (5 mg/kg body weight) by gastric gavage as a suspension in an Acacia gum-water vehicle (10 mg/mL). Animals were randomly distributed in three experimental groups: 1) controls only receiving vehicle; 2) M.F.A.S.C.W. 5 mg/kg body weight and 3) M.F.A.S.C.W. 100 mg/kg body weight. One hour after the administration of the substances, rats were anaesthetized using sodium pentobarbital (30 mg/kg) and the tail of the animals was submerged in a saline solution at 37° C., two (2) cm of the last portion of this tail were cutted and the bleeding time was measured using a digital chronometer.

Veinous thrombosis: Animals were randomly distributed in four experimental groups 1) controls; 2, 3 and 4 receiving M.F.A.S.C.W. at doses of 20, 100 and 200 mg/kg respectively.

Induction of thrombosis: All the treatments were administered orally and after an hour the animals were anaesthetized using sodium pentobarbital injected intraperitoneal route with hypotonic saline solution (0.22%) (1 ml/100 g of body weight), immediately their abdomens were opened and the cava vein was ligated in its upper part (2 cm) and part of this vein was collected in a filter paper and opened, extracting the thrombo that was transferred to a Petri's dishes with a filter paper humected in physiological saline solution standing at room temperature for an hour. This weight of the thrombo weas determined after that time.

The statistical comparison of results between treatment and control groups was carried out using the nonparametric Mann-Whitney U Test and for the analysis of the incidence was used the Fisher's Test.

Orally administered M.F.A.S.C.W. at dose of 100 mg/kg and 200 mg/kg significatively orevent veinous thrombosis but not the dose of 20 mg/kg. The results are shown in Table IV and V.

TABLE IV

Effect of M.F.A.S.C.W. over veinous thrombosis in rats ($\bar{x} \pm SD$)

Treatment	Dose (mg/kg)	n	Thrombo weight (mg)	Incidence of thrombos (%)
Control	—	20	4.12 ± 2.7	69
M.F.A.S.C.W.	20	11	5.24 ± 4.4	73.4
M.F.A.S.C.W	100	9	3.24 ± 3.2*	64.3
M.F.A.S.C.W	200	11	2.40 ± 1.3*	48

*$p < 0.05$ Mann-Whitney U Test

TABLE V

Effect of M.F.A.S.C.W. on the bleeding time of rats

Treatment	Dose (mg/kg)	n	Bleeding time (s)	
Control	—	15	215.8 ± 10.4	
M.F.A.S.C.W.	25	15	230.6 ± 25.5	ns
M.F.A.S.C.W.	100	13	249.0 ± 14.8	ns

ns: non significative.

As can be observed, the results demonstrated that M.F.A.S.C.W., in the range of doses assayed, diminished the size and weight of the thrombo in rats without increasing the bleeding time. These results demonstrated that M.F.A.S.C.W. shows anti-thrombotic effects without provoquing significative changes in blood coagulation, representing an advantage to diminish the effects of bleeding in the anti-thrombotic therapy and suggest that the mechanism of action could be related with an effect over the platelet rather than an inhibition of coagulation factors, between others.

EXAMPLE 9

To analyze the effect of the M.F.A.S.C.W. on experimentally induced gastric ulcers were studied two experimental models, in one the gastric ulcer is induced by ethanol and in the other by stress. Male and female Sprague Dawley rats weighing 200 to 250 g were adapted to laboratory conditions (25±2° C., ligth/darkness cycles of 12 h) with free access to water and food for 7 days. M.F.A.S.C.W. was prepared as a suspension in an Acacia gum/water vehicle. Animals were randomly distributed in different experimental groups and the administration of products was orally done using a gastric gavage (5 ml/kg body weight), the controls only received equivalent volumes of the vehicle. According to the experimental series the induction of gastric ulcer was induced by oral administration of ethanol (60%) or by stress. After a 24 hours fast period, gastric ulcers were induced in the animals.

Induction with ethanol: rats were randomly distributed in 4 experimental groups: 1) control and 2, 3 and 4) treated with M.F.A.S.C.W. at 25, 50 and 100 mg/kg respectively. An hour after treatment, each rat was administered 1 mL of ethanol by gastric gavage. One hour later, rats were sacrificed, their stomach were immediately removed, opened lengthwise along the greater curvature and washed with distilled water, then the damaged area was measured by means of a magnifying glass (x3).

Induction by stress: Animals were randomly distributed in three (3) experimental groups: 1) controls and 2 and 3 M.F.A.S.C.W. 50 and 200 mg/kg respectively. Immediately after treatment rats were individually immobilized in 5 cm diameter iron cylinders and submerged in a water bath at 24±1° C. up to the xyfoid level in order to produce stress in the rats. Animals were sacrificed 7 hours later, the stomachs were immediately removed, opened lengthwise along the greater curvature and washed with distilled water. The damaged areas were measured by two independent specialist in a double blind fashion. Lesions were evaluated as the total sumatory of the sizes of the damaged areas reported as mm of gastric ulcers.

All comparison between treatment and control groups were performed using the nonparametric Mann-Whitney U Test.

As is observed in Table VI, M.F.A.S.C.W. (100 mg/kg) significantly inhibited the occurence of gastric ulcers induced by ethanol.

TABLE VI

Effect of M.F.A.S.C.W. on gastric ulcers induced by ethanol (60%)

Treatment	Dose (mg/kg)	n	Ulcer size (mm)	Inhibition %
Control	—	13	16.2 ± 5.8	—
M.F.A.S.C.W.	25	10	10.9 ± 2.9	19
M.F.A.S.C.W.	50	9	8.4 ± 2.7	40
M.F.A.S.C.W.	100	10	5.0 ± 2.2*	64

*$p < 0.05$ (Mann-Whitney U Test)

In Table IV are shown the results of M.F.A.S.C.W. on gastric ulcers indiced by stress.

TABLE VII

Effect of M.F.A.S.C.W. on the gastric ulcers induced by stress (inmovilization and inmersion)

Treatment	Dose (mg/kg)	n	Ulcer's size (mm)
Control	—	14	46.1 ± 3.5
M.F.A.S.C.W.	50	6	44.9 ± 7.9
M.F.A.S.C.W.	200	6	26.8 ± 5.1**

*$p << 0.05$, **$p << 0.01$ Mann-Whitney U Test

As can be appreciated, M.F.A.S.C.W. protect the gastric mucose from lesions induced both by ethanol or by stress, being a model independent of the administered dose example 10.

Tablets containing 5 mg of M.F.A.S.C.W. were administered to a groups of patients affected with hypercholesteroleamia type II. Before these patients were included in the study, they suffered a 4 weeks of diet only period and only those having LDL-C values higher than 3.8 mmol/L were included in the study. Treatment with M.F.A.S.C.W. was maintained for 6 weeks. During this active treatment period, dietary conditions were maintained. After that period, the lipid profile was determined to each patient, results are reported in Tables VIII and IX.

TABLE VIII

Effect of MFASCW 5 mg on cholesterol and triglycerides serum levels

	Cholesterol (mmol/L)		Triglycerides (mmol/L)	
	Weeks			
Patient	0	6	0	6
1	6.46	6.05	2.15	2.04
2	6.40	5.41	2.77	2.04
3	7.48	6.17	1.71	2.10

TABLE VIII-continued

Effect of MFASCW 5 mg on cholesterol and triglycerides serum levels

	Cholesterol (mmol/L)		Triglycerides (mmol/L)	
	Weeks			
Patient	0	6	0	6
4	10.71	7.48	2.26	2.25
5	7.62	6.80	3.81	3.04
6	6.33	5.29	1.81	1.71
7	7.32	6.31	1.73	1.61
8	7.76	6.86	2.20	1.70
9	7.80	6.99	4.30	3.50
10	7.00	5.20	2.30	1.60
11	6.86	5.45	2.90	2.70
12	7.21	6.00	1.80	1.20
13	6.40	5.00	3.30	1.80
14	6.90	5.80	2.20	2.00
15	8.35	6.78	4.00	3.20
16	6.40	5.10	1.20	0.80
17	9.20	6.35	2.80	1.70
18	6.58	5.00	1.90	1.35
19	6.85	—	2.00	—
X	7.35	6.00	2.44	2.05
Δ%		18.2%		17.4%

TABLE IX

Effect of M.F.A.S.C.W. 5 mg on serum low density lipoproteins (LDL-C) as well as high density lipoproteins (HDL-C)

	LDL-C (mmol/L)		HDL-C (mmol/L)	
	Weeks			
Patient	0	6	0	6
1	5.25	5.02	1.00	1.60
2	4.91	4.92	0.85	1.20
3	6.77	6.20	1.50	1.61
4	8.45	9.01	1.35	1.40
5	3.48	—	0.75	1.20
6	9.95	8.85	1.12	1.08
7	7.70	—	1.01	1.10
8	4.42	4.27	0.90	1.30
9	4.72	5.24	—	—
10	6.18	4.64	—	—
11	5.47	5.34	0.88	0.98
12	4.04	3.66	1.15	1.20
13	5.25	5.04	1.89	1.65
14	6.31	4.33	1.05	1.56
15	4.78	4.25	0.68	0.85
16	5.51	5.16	1.30	1.20
17	5.40	5.02	1.12	1.60
18	5.60	4.96	0.98	0.96
19	5.20	—	0.78	—
X	5.40	3.98	1.08	1.28
Δ%		25.4%		20.7%

It can be observed that, after a 6 weeks treatment period, is produced a significative reduction of cholesterol ($p<0.001$), triglycerides ($p<0.01$) and LDL-C levels ($p<0.001$), as well as an significative increase ($p<0.05$) of HDL-C levels. The average reductions were of 18.2% (cholesterol), 17.4% (triglycerides), 25.7% (LDL-C) and the increase of HDL-C was of 20.7%.

These results demonstrate that treatment with M.F.A.S.C.W. is very effective, because in the 100% of the patients the final levels were lower than that of the beginning of the treatment and the variation percentages are bigger that the limits required by a drug to be considered as effective in the reduction of cholesterol and LDL-C.

What is claimed is:

1. A pharmaceutically beneficial composition of primary fatty acids consisting of a mixture of:

(a) 1-hexacosanoic acid;

(b) 1-octacosanoic acid;

(c) 1-nonacosanoic acid;

(d) 1-triacontanoic acid;

(e) 1-hentriacontanoic acid;

(f) 1-dotriacontanoic acid;

(g) 1-tritriacontanoic acid;

(h) 1-tetratriacontanoic acid;

(i) 1-pentatriacontanoic acid; and,

(j) 1-hexatriacontanoic acid;

wherein said pharmaceutically beneficial composition of primary fatty acids are a natural mixture obtained from a homogeneous saponification of sugar cane wax and substantially free of other fatty acids having 24 to 38 carbon atoms.

2. A pharmaceutically beneficial composition of primary fatty acids wherein said mixture comprises:

(a) 1-hexacosanoic acid which is about 0.3 to 1.5% of total weight;

(b) 1-octacosanoic acid which is about 25 to 50% of total weight;

(c) 1-nonacosanoic acid which is about 1 to 3% of total weight;

(d) 1-triacontanoic acid which is about 15 to 30% of total weight;

(e) 1-hentriacontanoic acid which is about 0.8 to 3% of total weight;

(f) 1-dotriacontanoic acid which is about 10 to 22% of total weight;

(g) 1-tritriacontanoic acid which is about 1 to 3% of total weight;

(h) 1-tetratriacontanoic acid which is about 10 to 22% of total weight;

(i) 1-pentatriacontanoic acid which is about 0.5 to 1.5% of total weight; and,

(j) 1-hexatriacontanoic acid which is about 2 to 9% of total weight wherein said pharmaceutically beneficial composition of primary fatty acids is a natural mixture obtained from a homogeneous saponification of sugar cane.

3. The pharmaceutically beneficial composition of primary fatty acids according to claim 2 wherein said composition further comprises:

an oral delivery device wherein said device is selected from the group consisting of tablets, dragees, and capsules wherein said mixture comprises about 0.5 to 25% by weight of said oral delivery device; and,

a delivery aid wherein said delivery aid is an acceptable pharmaceutical excipient wherein said delivery aid comprises the remaining percentage of total weight of said oral delivery device.

4. The pharmaceutically beneficial composition of primary fatty acids according to claim 3 wherein said acceptable pharmaceutical excipients are selected from the group consisting of fillers, agglutinants, disintegrators, lubricants, and sliders.

5. The pharmaceutically beneficial composition of primary fatty acids according to claim 3 wherein said oral delivery device is a tablet further comprising a mixture of lactose, corn starch, gelatin, sodium croscarmellose, talc, magnesium stearate and microcrystalline cellulose.

6. A method of treating medical conditions comprising the steps of:

diagnosing whether a medical condition exists which is treatable with primary fatty acids wherein said medical condition is selected from a group consisting of type II hypercholesterolemia, ischemia, thrombosis, platelet aggregation and gastric ulcers induced by compounds, agents or drugs; and,

administering a therapeutically beneficial dosage of a composition of primary fatty acids comprising a mixture of:

(a) 1-hexacosanoic acid;
(b) 1-octacosanoic acid;
(c) 1-nonacosanoic acid;
(d) 1-triacontanoic acid;
(e) 1-hentriacontanoic acid;
(f) 1-dotriacontanoic acid;
(g) 1-tritriacontanoic acid;
(h) 1-tetratriacontanoic acid;
(i) 1-pentatriacontanoic acid; and,
(j) 1-hexatriacontanoic acid

wherein said therapeutically beneficial dosage of a composition of primary fatty acids is a natural mixture obtained from a homogeneous saponification of sugar cane.

7. The method according to claim 6 wherein said medical condition is selected from a group consisting of type II hypercholesterolemia, ischemia, thrombosis, and platelet aggregation.

8. The method according to claim 7 wherein said therapeutically beneficial dosage of primary fatty acids is about 1 to about 100 mg daily.

9. The method according to claim 8 wherein said therapeutically beneficial dosage of primary fatty acids is administered orally or parenterally and is about 10 to 20 mg daily.

10. A method for obtaining a pharmaceutically beneficial composition of primary fatty acids mixture as free acids or in form of their salt from sugar cane wax comprising the steps of:

saponifying sugar cane wax with a concentrated hydroxide solution for at least 30 minutes wherein said concentrated hydroxide solution is selected from the group consisting of alkalines and earth alkalines;

isolating said fatty acid mixture from the rest of the components of said homogeneous saponification of sugar cane wax by extracting said components from said saponified wax using a solid-liquid extraction system, wherein said solid-liquid extraction system uses an extraction solvent wherein said extraction solvents are selected from the group consisting of ketones having 3 to 8 carbon atoms, alcohols having 1 to 5 carbon atoms, hydrocarbons having 5 to 8 carbon atoms, haloforms, aromatic compounds, mixtures and aqueous solutions thereof;

purifying the mixture by successive recrystallizations wherein successive recrystallizations are preformed in a recrystallization solution wherein said recrystallization solution is selected from the group consisting of ketones having 3 to 8 carbon atoms, alcohols having 1 to 5 carbon atoms, hydrocarbons having 5 to 8 carbon atoms, aromatic compounds, mixtures thereof, aqueous solutions thereof and water;

refluxing the components in an adequate reflux solvent wherein said reflux solvents are selected from the group consisting of ketones having 3 to 8 carbon atoms, alcohols having 1 to 5 carbon atoms, hydrocarbons having 5 to 8 carbon atoms, aromatic compounds, mixtures and aqueous solutions thereof;

hot filtering immediately thereafter the mixture; and,

regenerating the mixture using a regeneration solution wherein said regeneration solution is selected from the group consisting of organic acids, mineral acids, and combinations thereof wherein the regeneration produces the mixture comprising:

(a) 1-hexacosanoic acid;
(b) 1-octacosanoic acid;
(c) 1-nonacosanoic acid;
(d) 1-triacontanoic acid;
(e) 1-hentriacontanoic acid;
(f) 1-dotriacontanoic acid;
(g) 1-tritriacontanoic acid;
(h) 1-tetratriacontanoic acid;
(i) 1-pentatriacontanoic acid; and,
(j) 1-hexatriacontanoic acid.

11. The method according to claim 10:

wherein said concentrated hydroxide solution has a hydroxide weight of at least 5% to 25% of that of the weight of the wax to be processed;

wherein said saponification lasts for a period of at least 30 minutes and up to 5 hours;

wherein said extraction of said mixture ranges from 1 to 20 hours;

wherein said recrystallization ranges from 15 minutes up to 3 hours; and,

wherein said refluxing is performed between 15 minutes and 3 hours.

12. The method according to claim 8 wherein said concentrated hydroxide solution is selected from the group consisting of sodium, potassium and calcium.

13. The method according to claim 10 wherein said successive recrystallizations are preformed in a recrystallization solution which is selected from the group consisting of, methanol, ethanol, n-propanol, 2-propanol, n-butanol, n-pentanol, terbutanol, mixtures and aqueous solutions thereof;

wherein said extraction solvents are selected from the group consisting of methanol, ethanol, n-propanol, 2-propanol, n-butanol, n-pentanol, terbutanol, mixtures and aqueous solutions thereof;

wherein said reflux solvents are selected from the group consisting of methanol, ethanol, n-propanol, 2-propanol, n-butanol, n-pentanol, terbutanol, mixtures and aqueous solutions thereof.

14. The method according to claim 10 wherein said recrystallization solvent is selected from the group consisting of benzene, toluene, ethyl benzene, phenol, p-methyl toluene and mixtures thereof;

wherein said extraction solvent is selected from the group consisting of benzene, toluene, ethyl benzene, phenol p-methyl toluene and mixtures thereof;

wherein said reflux solvent which is selected from the group consisting of benzene, toluene, ethyl benzene, phenol p-methyl toluene and mixtures thereof.

15. The method according to claim 10 wherein said extraction solvent which is selected from the group consisting of chloroform, 1,2-dichloroethane, dichloromethane, trichloroethane, trichloromethane, 1,2,3-trichloropropane, and mixtures thereof.

16. The method according to claim 10 wherein said recrystallization solution is selected from the group consisting of hexane, pentane, isopentane, cyclohexane, heptane and mixtures thereof;

wherein said extraction solvent is selected from the group consisting of hexane, pentane, isopentane, cyclohexane, heptane and mixtures thereof;

wherein said reflux solvent is selected from the group consisting of hexane, pentane, isopentane, cyclohexane, heptane and mixtures thereof.

17. The method according to claim **10** wherein said recrystallization solution is water.

18. The method according to claim **10** wherein said regeneration solution is selected from the group consisting of hydrochloric acid, sulfuric acid, nitric acid, perchloric acid, acetic acid, oxalic acid and combinations thereof.

19. The method according to claim **10**:

wherein said recrystallization solution is selected from the group consisting of acetone, methyl butyl ketone, pentanone, methyl ethyl ketone, hexanone, mixtures thereof and aqueous solutions thereof;

wherein said extraction solution is selected from the group consisting of acetone, methyl butyl ketone, pentanone, methyl ethyl ketone, hexanone, and mixtures thereof;

wherein said reflux solvent is selected from the group consisting of acetone, methyl butyl ketone, pentanone, methyl ethyl ketone, hexanone and mixtures thereof.

* * * * *

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United States Patent [19]

Beeley et al.

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[54] **ANTI-DIABETIC PEPTIDES**

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[58] **Field of Search** 530/300, 308, 530/326; 514/2, 13; 424/184.1, 185.1

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[57] **ABSTRACT**

Compounds of formula I which act as amylin agonists with respect to certain desired amylin activities and as calcitonin agonists with respect to certain desired calcitonin activities are provided. Such compounds are useful in treating disturbances in fuel metabolism in mammals, including, but not limited to diabetes mellitus, including Type I diabetes and Type II diabetes. The present invention also relates to methods of treating Type I diabetes, treating Type II diabetes and to methods of beneficially regulating gastrointestinal motility comprising administration of a therapeutically effective among of one of the compounds. Also provided are pharmaceutical composition comprising a compound of formula I and a pharmaceutically acceptable carrier.

82 Claims, No Drawings

ANTI-DIABETIC PEPTIDES

FIELD OF THE INVENTION

The present invention is directed to novel peptide compounds for use as calcitonin agonists and as amylin agonists with respect to certain desired amylin activities. These compounds are useful in treating disturbances in fuel metabolism in mammals, including, but not limited to diabetes mellitus, including Type I diabetes and Type II diabetes.

BACKGROUND AND INTRODUCTION TO THE INVENTION

Diabetes mellitus is a serious metabolic disease that is defined by the presence of chronically elevated levels of blood glucose (hyperglycemia). This state of hyperglycemia is the result of a relative or absolute lack of activity of the peptide hormone, insulin. Insulin is produced and secreted by the β cells of the pancreas. Insulin is reported to promote glucose utilization, protein synthesis, and the formation and storage of carbohydrate energy as glycogen. Glucose is stored in the body as glycogen, a form of polymerized glucose, which may be converted back into glucose to meet metabolism requirements. Under normal conditions, insulin is secreted at both a basal rate and at enhanced rates following glucose stimulation, all to maintain metabolic homeostasis by the conversion of glucose into glycogen.

The term diabetes mellitus encompasses several different hyperglycemic states. These states include Type I (insulin-dependent diabetes mellitus or IDDM) and Type II (non-insulin dependent diabetes mellitus or NIDDM) diabetes. The hyperglycemia present in individuals with Type I diabetes is associated with deficient, reduced, or nonexistent levels of insulin which are insufficient to maintain blood glucose levels within the physiological range. Treatment of Type I diabetes involves administration of replacement doses of insulin, generally by a parental route. The hyperglycemia present in individuals with Type II diabetes is initially associated with normal or elevated levels of insulin; however, these individuals are unable to maintain metabolic homeostasis due to a state of insulin resistance in peripheral tissues and liver and, as the disease advances, due to a progressive deterioration of the pancreatic β cells which are responsible for the secretion of insulin. Thus, initial therapy of Type II diabetes may be based on diet and lifestyle changes augmented by therapy with oral hypoglycemic agents such as sulfonylureas. Insulin therapy is often required, however, especially in the latter states of the disease, in attempting to produce some control of hyperglycemia and minimize complications of the disease.

The structure and biology of amylin have previously been reviewed. See, for example, Young, *Current Opinion in Endocrinology and Diabetes*, 4:282–290 (1997); Gaeta and Rink, *Med. Chem. Res.*, 3:483–490 (1994); and, Pittner et al., *J. Cell. Biochem.*, 55S:19–28 (1994). Amylin is a 37 amino acid peptide hormone. It was isolated, purified and chemically characterized as the major component of amyloid deposits in the islets of pancreases of deceased human Type II diabetics (Cooper et al., *Proc. Natl. Acad. Sci. USA*, 84:8628–8632 (1987)). The amylin molecule has two important post-translational modifications: the C-terminus is amidated, i.e., the 37th residue is tyrosinamide, and the cysteines in positions 2 and 7 are cross-linked to form an intra-molecular N-terminal loop, both of which are essential for full biologic activity (Cooper et al., *Proc. Natl. Acad. Sci. USA*, 85:7763–7766 (1988)). Amylin is the subject of U.S. Pat. No. 5,367,052, issued Nov. 22, 1994, in the names of Garth Cooper and Antony Willis, who discovered the hormone.

In Type I diabetes and late stage Type II diabetes, amylin has been shown to be deficient and combined replacement with insulin has been proposed as a preferred treatment over insulin alone in all forms of insulin-dependent diabetes. The use of amylin and amylin agonists for the treatment of diabetes mellitus is the subject of U.S. Pat. No. 5,175,145, issued Dec. 29, 1992. Pharmaceutical compositions containing amylin and amylin plus insulin are described in U.S. Pat. No. 5,124,314, issued Jun. 23, 1992.

Excess amylin action has been said to mimic key features of the earlier stages of Type II diabetes and amylin blockade has been proposed as a novel therapeutic strategy. It has been disclosed in U.S. Pat. No. 5,266,561, issued Nov. 30, 1993, that amylin causes reduction in both basal and insulin-stimulated incorporation of labeled glucose into glycogen in skeletal muscle. The treatment of Type II diabetes and insulin resistance with amylin antagonists is disclosed.

Amylin is primarily synthesized in pancreatic beta cells and is secreted in response to nutrient stimuli such as glucose and arginine. Studies with cloned beta-cell tumor lines (Moore et al., *Biochem. Biophys. Res. Commun.*, 179(1) (1991)) and perfused rat pancreases (Ogawa et al., *J. Clin. Invest.*, 85:973–976 (1990)) have shown that short pulses, 10 to 20 minutes, of nutrient secretagogues such as glucose and arginine, stimulate release of amylin as well as insulin. The molar amylin:insulin ratio of the secreted proteins varies between preparations from about 0.01 to 0.4, but appears not to vary much with acute stimuli in any one preparation. However, during prolonged stimulation by elevated glucose, the amylin:insulin ratio can progressively increase (Gedulin et al., *Biochem. Biophys. Res. Commun.*, 180(1):782–789 (1991)). Thus, amylin and insulin are not always secreted in a constant ratio.

It has been discovered and reported that certain actions of amylin are similar to non-metabolic actions of CGRP and calcitonin; however, the metabolic actions of amylin discovered during investigations of this recently identified protein appear to reflect its primary biological role. At least some of these metabolic actions are mimicked by CGRP, albeit at doses which are markedly vasodilatory (see, e.g., Leighton and Cooper, *Nature*, 335:632–635 (1988)); Molina et al., *Diabetes*, 39:260–265 (1990)).

It is believed that amylin acts through receptors present in plasma membranes. Studies of amylin and CGRP, and the effect of selective antagonists, have led to reports that amylin acts via its own receptor (Beaumont et al., *Br. J. Pharmacol.*, 115(5):713–715 (1995); Wang et al., *FEBS Letts.*, 219:195–198 (1991 b)), in contrast to the conclusion of other workers that amylin may act primarily at CGRP receptors (e.g, Chantry et al., *Biochem. J.*, 277:139–143 (1991)); Zhu et al., *Biochem. Biophys. Res. Commun.*, 177(2):771–776 (1991)). Amylin receptors and their use in methods for screening and assaying for amylin agonist and antagonist compounds are described in U.S. Pat. No. 5,264,372, issued Nov. 23, 1993.

Amylin and amylin agonists have also been shown to suppress glucagon secretion. When influences that would otherwise affect glucagon secretion were controlled (plasma glucose, insulin and blood pressure), amylin reportedly suppressed the glucagon response to arginine in rats. Gedulin et al., *Metabolism*, 46:67–70 (1997). The amylin anaogue, pramlintide (^{25,28,29}Pro-human amylin), has been reported to eliminate the post-prandial surge in glucagon

concentration in subjects with Type I diabetes (Fineman et al., *Diabetes*, 40:30A (1997)). Pramlintide, and other amylin agonist analogues, are described and claimed in U.S. Pat. No. 5,686,411, issued Nov. 11, 1997.

Amylin and amylin agonists potently inhibit gastric emptying in rats (Young et al., *Diabetologia* 38(6):642–648 (1995)), dogs (Brown et al., *Diabetes* 43(Suppl 1):172A (1994)) and humans (Macdonald et al., *Diabetologia* 38(Suppl 1):A32 (abstract 118)(1995)). Gastric emptying is reportedly accelerated in amylin-deficient Type I diabetic BB rats (Young et al., *Diabetologia*, supra; Nowak et al., *J. Lab. Clin. Med.*, 123(1):110–6 (1994)) and in rats treated with the amylin antagonist, AC187 (Gedulin et al., *Diabetologia*, 38(Suppl 1):A244 (1995). The effect of amylin on gastric emptying appears to be physiological (operative at concentrations that normally circulate).

Non-metabolic actions of amylin include vasodilator effects which may be mediated by interaction with CGRP vascular receptors. Reported in vivo tests suggest that amylin is at least about 100 to 1000 times less potent than CGRP as a vasodilator (Brain et al., *Eur. J. Pharmacol.*, 183:2221 (1990); Wang et al., *FEBS Letts.*, 291:195–198 (1991)).

Injected into the brain, or administered peripherally, amylin has been reported to suppress food intake, e.g., Chance et al., *Brain Res.*, 539:352–354 (1991)), an action shared with CGRP and calcitonin. The effective concentrations at the cells that mediate this action are not known. Amylin has also been reported to have effects both on isolated osteoclasts where it caused cell quiescence, and in vivo where it was reported to lower plasma calcium by up to 20% in rats, in rabbits, and in humans with Paget's disease (see, e.g., Zaidi et al., *Trends in Endocrinal.* and *Metab.*, 4:255–259 (1993). From the available data, amylin appears to be 10 to 30 times less potent than human calcitonin for these actions. Interestingly, it was reported that amylin increased osteoclast cAMP production but not cytosolic Ca^{2+}, while calcitonin does both (Alam et al., *Biochem. Biophys. Res. Commun.*, 179(1):134–139 (1991)). It was suggested, though not established, that amylin may act via a single receptor type whereas calcitonin may act via two receptors, one of which may be common to amylin activity.

It has also been discovered that, surprisingly in view of its previously described renal vasodilator and other properties, amylin markedly increases plasma renin activity in intact rats when given subcutaneously in a manner that avoids any disturbance of blood pressure. This latter point is important because lowered blood pressure is a strong stimulus to renin release. Amylin antagonists, such as amylin receptor antagonists, including those selective for amylin receptors compared to CGRP and/or calcitonin receptors, can be used to block the amylin-evoked rise of plasma renin activity. The use of amylin antagonists to treat renin-related disorders is described in U.S. Pat. No. 5,376,638, issued Dec. 27, 1994.

In normal humans, fasting amylin levels from 1 to 10 pM and post-prandial or post-glucose levels of 5 to 20 pM have been reported (see, e.g., Koda et al., *The Lancet*, 339:1179–1180 (1992)). In obese, insulin-resistant individuals, post-food amylin levels can go higher, reaching up to about 50 pM. For comparison, the values for fasting and post-prandial insulin are 20 to 50 pM, and 100 to 300 pM respectively in healthy people, with perhaps 3- to 4-fold higher levels in insulin-resistant people. In Type I diabetes, where beta cells are destroyed, amylin levels are at or below the levels of detection and do not rise in response to glucose (Koda et al., *The Lancet*, 339:1179–1180 (1992)). In normal mice and rats, basal amylin levels have been reported from 30 to 100 pM, while values up to 600 pM have been measured in certain insulin-resistant, diabetic strains of rodents (e.g., Huang et al., *Hypertension*, 19:I-101–I-109 (1991)).

In mammals, calcitonin functions in the regulation of bone marrow turnover and calcium metabolism. Calcitonin, which is caused to be released from the thyroid by elevated serum calcium levels, produces actions on bone and other organs which tend to reduce serum calcium levels. Calcitonin inhibits osteoclast activity and reduces bone resorption, thereby reducing serum calcium levels. Calcitonin also alters calcium, phosphate and electrolyte excretion by the kidney, although the physiological significance of this is not reported. Calcitonin has been used clinically for treatment of disorders of calcium metabolism and pain, and its relationship to increased glucose levels in mammals has been the subject of varying reports. See, e.g., Azria et al., "Calcitonins—Physiological and Pharmacological Aspects," pp. 24–25 (Springer-Verlag 1989). The use of calcitonins in the treatment of diabetes mellitus is described in U.S. Pat. No. 5,321,008, issued Jun. 14, 1994, and in U.S. Pat. No. 5,508,260, issued Apr. 16, 1996. Certain compounds reported to be calcitonin derivatives have been said to lower the calcium plasma level and to influence bone metabolism in U.S. Pat. No. 4,758,550, issued Jul. 19, 1988.

SUMMARY OF THE INVENTION

The present invention provides novel compounds for use in regulating certain metabolic effects mediated by amylin and calcitonin in mammals. Surprisingly, these compounds act as amylin agonists for certain of amylin's effects and as calcitonin agonists for certain of calcitonin's effects.

Among other factors, the present invention is based on our unexpected discovery that the compounds of the present invention exhibit a biological profile which includes acting as agonists for certain of the effects of calcitonin and amylin. In particular these compounds act as amylin agonists in the inhibition of gastric emptying. Due to their surprising combination of biological effects, these compounds will be useful in treating diabetes, including Type I diabetes and insulin dependent (late stage) Type II diabetes, due in part to their effects on inhibiting gastric emptying. Applicants note that the compounds of the present invention exhibit advantageous biological activity and yet are peptide amides which are less than about half to about two-fifths the size of amylin. Due to their smaller size and molecular weight, these compounds are easier and more economical to synthesize. In addition, these compounds will be more amenable to drug delivery via patch technology, microsphere technology and/or buccal technology, among others. Applicants note that previously reported agonists of amylin were substantially full length. See, e.g., U.S. Pat. No. 5,686,411.

According to the present invention, provided are compounds of the formula I:

X_1-Xaa_1-X_2-Xaa_2-X_3-Xaa_3-X_4-Xaa_4-X_5-Xaa_5-X_6-NH_2 wherein X_1 is Lys, Arg or absent;

X_2 is $Xaa_6Xaa_7Xaa_8Xaa_9$ (SEQ. ID. NO. 47) or Z-Xaa_{10}SerThr, provided that if X_2 is Z-Xaa_{10}Ser-Thr, then X_1 and Xaa_1 are both absent;

X_3 is AlaThr, AlaSer, SerMet, GluThr or ValThr;

X_4 is ArgLeuAla, HisLeuAla, ArgIleAla, LysIleAla, ArgMetAla, HisMetAla, LysMetAla or ArgLeuThr;

X_5 is PheLeu, PheIle, PheMet, TyrLeu, TyrIle, TyrMet, TrpMet, TrpIle or TrpMet;

X_6 is ArgSerSerGlyTyr (SEQ. ID. NO. 48), LysSerSerGlyTyr (SEQ. ID. NO. 49), HisSerSerGlyTyr (SEQ. ID.

NO. 50), ProSerSerGlyTyr (SEQ. ID. NO. 51), ArgSerArgGlyTyr (SEQ. ID. NO. 52), ArgThrSerGlyTyr (SEQ. ID. NO. 53), ArgAlaSerGlyTyr (SEQ. ID. NO. 54), AlaSerSerGlyTyr (SEQ. ID. NO. 55), ArgSerAlaGlyTyr (SEQ. ID. NO. 56), HisSerAlaGlyTyr (SEQ. ID. NO. 57), ArgSerGlyTyr (SEQ. ID. NO. 58), ArgSer, LysSer, HisSer, ArgThr, ProSer or Arg;

Xaa_1 is Cys or absent;

Xaa_2 is Cys or Ala;

Xaa_3 is Gln, Ala or Asn;

Xaa_4 is Asn, Ala or Gln;

Xaa_5 is Val, Ala, Ile, Met, Leu, PentylGly, or t-butylGly;

Xaa_6 is Asn, Gln or Asp;

Xaa_7 is Thr, Ser, Met, Val, Leu or Ile;

Xaa_8 is Ala or Val;

Xaa_9 is Thr or Ser;

Xaa_{10} is Leu, Val, Met or Ile; and

Z is an alkanoyl group of about 1 to about 8 carbon atoms or absent;

and pharmaceutically acceptable salts thereof. Methods of using compounds of the present invention in the treatment of various conditions which would benefit such conditions, such as diabetes and gastrointestinal disorders, as well as pharmaceutical compositions containing compounds of the present invention, are also described and claimed herein.

Definitions

In accordance with the present invention and as used herein, the following terms are defined to have the following meanings, unless explicitly stated otherwise.

The term "amylin" is understood to include the human peptide hormone amylin secreted from the beta cells of the pancreas.

"Amylin agonist" is also a term known in the art, and refers to a compound which has one or more biological activities of amylin. An amylin agonist may be a peptide or a non-peptide compound. Such compounds may act as amylin agonists, normally, it is presently believed, by virtue of binding to or otherwise directly or indirectly interacting with an amylin receptor or other receptor or receptors with which amylin itself may interact to elicit a biological response.

The term "amylin antagonist" refers to a compound which inhibits one or more effects of amylin. An amylin antagonist may be a peptide or non-peptide compound.

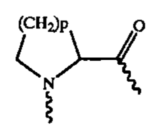
The term "alkanoyl" refers to the group RC(=O)— wherein R is a straight chain or branched chain alkyl group, which may be derived from a corresponding carboxylic acid.

The term "amino acid" refers to natural amino acids, unnatural amino acids, and amino acid analogs, all in their D and L stereoisomers if their structure allow such stereoisomeric forms. Natural amino acids include alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), Lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), typtophan (Trp), tyrosine (Tyr) and valine (Val). Unnatural amino acids include, but are not limited to azetidinecarboxylic acid, 2-aminoadipic acid, 3-aminoadipic acid, beta-alanine, aminopropionic acid, 2-aminobutyric acid, 4-aminobutyric acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2-aminoisobutyric acid, 3-aminoisbutyric acid, 2-aminopimelic acid, tertiary-butylglycine, 2,4-diaminoisobutyric acid, desmosine, 2,2'-diaminopimelic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-ethylasparagine, homoproline,

hydroxylysine, allo-hydroxylysine, 3-hydroxyproline, 4-hydroxyproline, isodesmosine, allo-isoleucine, N-methylalanine, N-methylglycine, N-methylisoleucine, N-methylpentylglycine, N-methylvaline, naphthalanine, norvaline, norleucine, ornithine, pentylglycine, pipecolic acid and thioproline. Amino acid analogs include the natural and unnatural amino acids which are chemically blocked, reversibly or irreversibly, or modified on their N-terminal amino group or their side-chain groups, as for example, methionine sulfoxide, methionine sulfone, S-(carboxymethyl)-cysteine, S-(carboxymethyl)-cysteine sulfoxide and S-(carboxymethyl)-cysteine sulfone.

The term "amino acid analog" refers to an amino acid wherein either the C-terminal carboxy group, the N-terminal amino group or side-chain functional group has been chemically codified to another functional group. For example, aspartic acid-(beta-methyl ester) is an amino acid analog of aspartic acid; N-ethylglycine is an amino acid analog of glycine; or alanine carboxamide is an amino acid analog of alanine.

The term "amino acid residue" refers to radicals having the structure: (1) —C(O)—R—NH— or —NH—R—C(O)— wherein R typically is —CH(R')—, wherein R' is an amino acid side chain, typically H or a carbon containing substitutent; or (2),



wherein p is 1, 2 or 3 representing the azetidinecarboxylic acid, proline or pipecolic acid residues, respectively. "Calcitonin agonist" refers to a compound which has one or more biological activities of calcitonin. A calcitonin agonist may be a peptide or non-peptide compound.

The term "lower" referred to herein in connection with organic radicals such as alkyl groups defines such groups with up to and including about 6, preferably up to and including 4 and advantageously one or two carbon atoms. Such groups may be straight chain or branched chain. "Pharmaceutically acceptable salt" includes salts of the compounds described herein derived from the combination of such compounds and an organic or inorganic acid. In practice the use of the salt form amounts to use of the base form. The compounds are useful in both free base and salt form.

In addition, the following abbreviations stand for the following:

"ACN" or "CH_3CN" refers to acetonitrile.

"Boc", "tBoc" or "Tboc" refers to t-butoxy carbonyl.

"DCC" refers to N,N'-dicyclohexylcarbodiimide.

"Fmoc" refers to fluorenylmethoxycarbonyl.

"HBTU" refers to 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexaflurophosphate.

"HOBt" refers to 1-hydroxybenzotriazole monohydrate.

"homoP" or hPro" refers to homoproline.

"MeAla" or "Nme" refers to N-methylalanine.

"naph" refers to naphthylalanine.

"Pg" or pGly" refers to pentylglycine.

"tBuG" refers to tertiary-butylglycine.

"ThioP" or tPro" refers to thioproline.

3Hyp" refers to 3-hydroxyproline

4Hyp" refers to 4-hydroxyproline

NAG" refers to N-alkylglycine

NAPG" refers to N-alkylpentylglycine

"Norval" refers to norvaline

"Norleu" refers to norleucine

DETAILED DESCRIPTION OF THE INVENTION

Preferred Compounds

According to the present invention, provided are compounds of formula I:

X_1-Xaa_1-X_2-Xaa_2-X_3-Xaa_3-X_4-Xaa_4-X_5-Xaa_5-X_6-NH_2

wherein X_1 is Lys, Arg or absent;

X_2 is $Xaa_6Xaa_7Xaa_8Xaa_9$ (SEQ. ID. NO. 47) or Z-Xaa_{10}SerThr, provided that if X_2 is Z-Xaa_{10}Ser-Thr, then X_1 and Xaa_1 are both absent;

X_3 is AlaThr, AlaSer, SerMet, GluThr or ValThr;

X_4 is ArgLeuAla, HisLeuAla, ArgIleAla, LysIleAla, ArgMetAla, HisMetAla, LysMetAla or ArgLeuThr;

X_5 is PheLeu, PheIle, PheMet, TyrLeu, TyrIle, TyrMet, TrpMet, TrpIle or TrpMet;

X_6 is ArgSerSerGlyTyr (SEQ. ID. NO. 48), LysSerSerGlyTyr (SEQ. ID. NO. 49), HisSerSerGlyTyr (SEQ. ID. NO. 50), ProSerSerGlyTyr (SEQ. ID. NO. 51), ArgSerArgGlyTyr (SEQ. ID. NO. 52), ArgThrSerGlyTyr (SEQ. ID. NO. 53), ArgAlaSerGlyTyr (SEQ. ID. NO. 54), AlaSerSerGlyTyr (SEQ. ID. NO. 55), ArgSerAlaGlyTyr (SEQ. ID. NO. 56), HisSerAlaGlyTyr (SEQ. ID. NO. 57), ArgSerGlyTyr (SEQ. ID. NO. 58), ArgSer, LysSer, HisSer, ArgThr, ProSer or Arg; and

Xaa_1 is Cys or absent;

Xaa_2 is Cys or Ala;

Xaa_3 is Gln, Ala or Asn;

Xaa_4 is Asn, Ala or Gln;

Xaa_5 is Val, Ala, Ile, Met, Leu, PentylGly, or t-butylGly;

Xaa_6 is Asn, Gln or Asp;

Xaa_7 is Thr, Ser, Met, Val, Leu or Ile;

Xaa_8 is Ala or Val;

Xaa_9 is Thr or Ser;

Xaa_{10} is Leu, Val, Met or Ile; and

Z is an alkanoyl group of about 1 to about 8 carbon atoms or absent;

and pharmaceutically acceptable salts thereof.

Preferred are compounds wherein X_1 is Lys or absent.

Preferred X_2 groups include $Xaa_6Xaa_7Xaa_8Xaa_9$ wherein Xaa_6 is Asn, Xaa_8 is Ala, and Xaa_9 is Thr or Z-Xaa_{10}SerThr wherein Xaa_{10} is Leu, Val or Met. When X_2 is $Xaa_6Xaa_7Xaa_8Xaa_9$, preferred X_2 groups include AsnThrAlaThr (SEQ. ID. NO. 59), AsnValAlaThr (SEQ. ID. NO. 60), AsnLeuAlaThr (SEQ.ID. NO. 61) and AsnMetAlaThr (SEQ. ID. NO. 62). When X_2 is Z-Xaa_{10}SerThr, especially preferred are compounds wherein Xaa_{10} is Leu.

Preferred X_3 groups include AlaThr.

Preferred X_4 groups include ArgLeuAla.

Preferred X_5 groups include PheLeu.

Preferred X_6 groups include ArgSerSerGlyTyr (SEQ. ID. NO. 48), HisSerSerGlyTyr (SEQ. ID. NO. 50), ArgSer and HisSer. Especially preferred are ArgSerSerGlyTyr (SEQ. ID. NO. 48) and ArgSer.

Preferred are compounds wherein Xaa_3 is Gln or Ala.

Preferred are compounds wherein Xaa_4 is Asn or Ala.

Preferred are compounds wherein Xaa_5 is Val or Ala.

Preferred Z groups include alkanoyl groups having about 3 to about 6 carbon atoms.

Preferred compounds include those where Xaa_1 and Xaa_2 are Cys. According to an especially preferred aspect, the two cysteines may advantageously form a disulfide bridge.

According to one preferred aspect, preferred compounds include those compounds wherein Xaa_3 is Ala. Particularly preferred are compounds wherein Xaa_4 and Xaa_5 are Ala. Alternatively, particularly preferred compounds include compounds wherein Xaa_4 is Asn and Xaa_5 is Val.

Preferred compounds according to an alternately preferred aspect include compounds wherein Xaa_3 is Gln, Xaa_4 is Asn and Xaa_5 is Val.

According to an alternate aspect, compounds are provided wherein X_3 is AlaThr, X_4 is ArgLeuAla and X_5 is PheLeu. Particularly preferred are compounds wherein X_6 is ArgSerSerGlyTyr (SEQ. ID. NO. 48), HisSerSerGlyTyr (SEQ. ID. NO. 60), ArgSer OR HisSer. According to one especially preferred aspect, X_6 is ArgSerSerGlyTyr (SEQ. ID. NO. 48) or ArgSer. According to an alternately preferred aspect, X_6 is ArgSerSerGlyTyr (SEQ. ID. NO. 48) or HisSerSerGlyTyr (SEQ. ID. NO. 50). Preferred compounds include those wherein Xaa_1 is Cys and especially preferred compounds include those wherein Xaa_2 is Cys. Where Xaa_1 and Xaa_2 are both Cys, they may advantageously form a disulfide bridge. Preferably X_2 is AsnThrAlaThr (SEQ. ID. NO. 59), AsnValAlaThr (SEQ. ID. NO. 60), AsnLeuAlaThr (SEQ. ID. NO. 61), or AsnMetAlaThr (SEQ. ID. NO. 62). Preferred are compounds wherein Xaa_3 is Ala or Glu, wherein Xaa_4 is Ala or Asn and wherein Xaa_5 is Ala or Val. According to this aspect, especially preferred compounds include those wherein X_2 is AsnThrAlaThr (SEQ. ID. NO. 59) or AsnValAlaThr (SEQ. ID. NO. 60), and X_6 is ArgSerSerGlyTyr (SEQ. ID. NO. 48). Particularly preferred compounds according to this aspect include those wherein X_2 is AsnThrAlaThr (SEQ. ID. NO. 59) and Xaa_3, Xaa_4, and Xaa_5 are Ala; especially preferred are those compounds wherein X_1 is Lys. Alternately, particularly preferred compounds according to this aspect include compounds wherein X_6 is ArgSer or HisSer, more preferably ArgSer; preferably X_2 is AsnValAlaThr (SEQ. ID. NO. 60), Xaa_3 is Gln, Xaa_4 is Asn, Xaa_5 is Val and X_1 is absent.

According to an alternate aspect, preferred compounds include those wherein X_1 is absent. Especially preferred are compounds wherein Xaa_1 is absent. According to this aspect, preferred X_6 groups include ArgSer and HisSer, more preferably ArgSer. Particularly preferred compounds according to this aspect include those wherein X_2 is Z-Xaa_{10}SerThr, preferably Xaa_{10} is Leu, Val or Met. Preferred compounds include those where Xaa_3 is Ala, Xaa_4 is Ala and Xaa_5 is Ala.

Preferred peptide compounds of the present invention include those having an amino acid sequence selected from SEQ. ID. NOS. 1 to 46. Particularly preferred are those having an amino acid sequence selected from SEQ. ID. NOS. 1 to 16. Especially preferred peptide compounds include those having an amino acid sequence selected from SEQ. ID. NOS. 1 to 6.

Also particularly preferred are compounds wherein (a) X_1 is Lys or absent and, Xaa_1 is Cys and X_2 is AsnThrAlaThr (SEQ. ID. NO. 59) or AsnValAlaThr (SEQ. ID. NO. 60) or (b) X_1 and Xaa_1 are absent and X_2 is Z-LeuSerThr. If Xaa_1 is absent, then Xaa_2 is preferably Ala. If Xaa_1 is Cys, then Xaa_2 is preferably Cys and Xaa_1 and Xaa_2 form a disulfide bridge. Especially preferred compounds include those described in Examples 1 to 6 (SEQ. ID. NOS. 1 to 6).

Preparation of Compounds

The compounds described herein may be prepared using standard solid-phase peptide synthesis techniques and pref-

erably an automated or semiautomated peptide synthesizer. Typically, using such techniques, an α-N-carbamoyl protected amino acid and an amino acid attached to the growing peptide chain on a resin are coupled at room temperature in an inert solvent such as dimethylformamide, N-methylpyrrolidinone or methylene chloride in the presence of coupling agents such as dicyclohexylcarbodiimide and 1-hydroxybenzotriazole in the presence of a base such as diisopropylethylamine. The α-N-carbamoyl protecting group is removed from the resulting peptide-resin using a reagent such as trifluoroacetic acid or piperidine, and the coupling reaction repeated with the next desired N-protected amino acid to be added to the peptide chain. Suitable N-protecting groups are well known in the art, with t-butyloxycarbonyl (tBoc) and fluorenylmethoxycarbonyl (Fmoc) being preferred herein.

The solvents, amino acid derivatives and 4-methylbenzhydryl-amine resin used in the peptide synthesizer may be purchased from Applied Biosystems Inc. (Foster City, Calif.). The following side-chain protected amino acids may be purchased from Applied Biosystems, Inc.: Boc-Arg(Mts), Fmoc-Arg(Pmc), Boc-Thr(Bzl), Fmoc-Thr(t-Bu), Boc-Ser(Bzl), Fmoc-Ser(t-Bu), Boc-Tyr(BrZ), Fmoc-Tyr(t-Bu), Boc-Lys(Cl-Z), Fmoc-Lys(Boc), Boc-Glu (Bzl), Fmoc-Glu(t-Bu), Fmoc-His(Trt), Fmoc-Asn(Trt), and Fmoc-Gln(Trt). Boc-His(BOM) may be purchased from Applied Biosystems, Inc. or Bachem Inc. (Torrance, Calif.). Anisole, dimethylsulfide, phenol, ethanedithiol, and thioanisole may be obtained from Aldrich Chemical Company (Milwaukee, Wis.). Air Products and Chemicals (Allentown, Pa.) supplies HF. Ethyl ether, acetic acid and methanol may be purchased from Fisher Scientific (Pittsburgh, Pa.).

Solid phase peptide synthesis may be carried out with an automatic peptide synthesizer (Model 430A, Applied Biosystems Inc., Foster City, Calif.) using the NMP/HOBt (Option 1) system and tBoc or Fmoc chemistry (see, Applied Biosystems User's Manual for the ABI 430A Peptide Synthesizer, Version 1.3B Jul. 1, 1988, section 6, pp. 49–70, Applied Biosystems, Inc., Foster City, Calif.) with capping. Boc-peptide-resins may be cleaved with HF (−5° C. to 0° C., 1 hour). The peptide may be extracted from the resin with alternating water and acetic acid, and the filtrates lyophilized. The Fmoc-peptide resins may be cleaved according to standard methods (*Introduction to Cleavage Techniques*, Applied Biosystems, Inc., 1990, pp. 6–12). Peptides may be also be assembled using an Advanced Chem Tech Synthesizer (Model MPS 350, Louisville, Ky.).

Peptides may be purified by RP-HPLC (preparative and analytical) using a Waters Delta Prep 3000 system. A C4, C8 or C18 preparative column (10 μ, 2.2×25 cm; Vydac, Hesperia, Calif.) may be used to isolate peptides, and purity may be determined using a C4, C8 or C18 analytical column (5 μ, 0.46×25 cm; Vydac). Solvents (A=0.1% TFA/water and B=0.1% TFA/CH_3CN) may be delivered to the analytical column at a flowrate of 1.0 ml/min and to the preparative column at 15 ml/min. Amino acid analyses may be performed on the Waters Pico Tag system and processed using the Maxima program. Peptides may be hydrolyzed by vapor-phase acid hydrolysis (115° C., 20–24 h). Hydrolysates may be derivatized and analyzed by standard methods (Cohen, et al., *The Pico Tag Method: A Manual of Advanced Techniques for Amino Acid Analysis*, pp. 11–52, Millipore Corporation, Milford, Mass. (1989)). Fast atom bombardment analysis may be carried out by M-Scan, Incorporated (West Chester, Pa.). Mass calibration may be performed using cesium iodide or cesium iodide/glycerol. Plasma desorption ionization analysis using time of flight detection may be carried out on an Applied Biosystems Bio-Ion 20 mass spectrometer. Electrospray mass spectroscopy may be carried out on a VG-Trio machine.

Peptide compounds useful in the invention may also be prepared using recombinant DNA techniques, using methods now known in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d Ed., Cold Spring Harbor (1989).

Non-peptide compounds useful in preparing compounds of the present invention may be prepared by art-known methods. For example, phosphate-containing amino acids and peptides containing such amino acids, may be prepared using methods known in the art. See, e.g., Bartlett and Landen, *Biorg. Chem.* 14:356–377 (1986).

The compounds referenced above may form salts with various inorganic and organic acids and bases. Such salts include salts prepared with organic and inorganic acids, for example, HCl, HBr, H_2SO_4, H_3PO_4, trifluoroacetic acid, acetic acid, formic acid, methanesulfonic acid, toluenesulfonic acid, maleic acid, fumaric acid succinic acid and tartaric acid and camphorsulfonic acid. Salts prepared with bases include ammonium salts, alkali metal salts, e.g., sodium and potassium salts, and alkali earth salts, e.g., calcium and magnesium salts. Acetate, hydrochloride, and trifluoroacetate salts are preferred. The salts may be formed by conventional means, as by reacting the free acid or base forms of the product with one or more equivalents of the appropriate base or acid in a solvent or medium in which the salt is insoluble, or in a solvent such as water which is then removed in vacuo or by freeze-drying or by exchanging the ions of an existing salt for another ion on a suitable ion exchange resin.

Biological Activity

Activities of the compounds of the present invention are evaluated and/or quantified with various screening assays, including the nucleus accumbens receptor binding assay described in Example A, the C1a agonist assay described in Example B, the C1a binding assay described in Example C and the gastric emptying assay described in Example D.

The nucleus accumbens receptor binding assay, a competition assay which measures the ability of compounds to bind specifically to membrane-bound amylin receptors, is described in U.S. Pat. No. 5,264,372, issued Nov. 23, 1993, the disclosure of which is incorporated herein by reference. The nucleus accumbens receptor binding assay is also described in Example A below. A preferred source of the membrane preparations used in the assay is the basal forebrain which comprises membranes from the nucleus accumbens and surrounding regions. Compounds being assayed compete for binding to these receptor preparations with radiolabeled ^{125}I Bolton Hunter rat amylin. Competition curves, wherein the amount bound (B) is plotted as a function of the log of the concentration of ligand are analyzed by computer, using analyses by nonlinear regression to a 4-parameter logistic equation (Inplot program; GraphPAD Software, San Diego, Calif.) or the ALLFIT program of DeLean et al. (ALLFIT, Version 2.7 (NIH, Bethesda, Md. 20892)). Munson and Rodbard, *Anal. Biochem.* 107:220–239 (1980). The results are reported in Table I.

Peptide compounds of the present invention may be evaluated for agonist activity using the procedures described in Example B. The plasma membrane preparation of a 7-transmembrane-G-protein coupled receptor (GPCR) contains not only the receptor but also the G-proteins which constitute the first step in the intracellular signal transduction process when the receptor is activated by an agonist

ligand. These G-proteins have a guanine nucleotide binding site, normally occupied by GDP in the resting or inactive conformation. Agonist activation of a GPCR is accompanied by displacement of GDP by GTP from this site. Thus, measurement of the binding of a radiolabelled ligand, namely [^{35}S]-GTPγS, for this binding site constitutes a measure of agonist potency for a given ligand. For agonism at the C1a receptor, a similar membrane preparation to that described below for the C1a binding studies is used. C1a pcDNA construction and transfection were carried out as previously described (Albrandt et al. 1993, FEBS Letters, 325:225–232). HEK293 cell lines showing stable expression of the rat C1a-type calcitonin receptor (C1a/293) or the human C1a-type calcitonin receptor (1154/293) were selected by G418 resistance and limiting dilution culture methods. Plasma membranes were collected from homogenized HEK293 cells and used in the [^{35}S]-GTPγS assay. Individual test peptides at concentrations spanning 6 log units starting at around 10^{-5}M were examined for their ability to bind [^{35}S]-GTPγS. Maximum agonist-specific binding was measured in the presence of 1 μM human calcitonin, constitutive binding was measured in the presence of buffer alone. Peptide potencies (EC_{50}'s for concentration response curves) were calculated by non-linear regression using Prism™ (version 2.01, GraphPAD Software, San Diego, Calif.). The results are reported in Table I.

Peptide compounds of the present invention may be evaluated for binding to the C1a receptor using the procedures described in Example C. The C1a receptor is the predominant mammalian calcitonin receptor subtype. C1a pcDNA construction and transfection were carried out as previously described (Albrandt et al. 1993, FEBS Letters, 325:225–232). HEK293 cell lines showing stable expression of the rat C1a-type calcitonin receptor (C1a/293) or the human C1a-type calcitonin receptor (1154/293) were selected by G418 resistance and limiting dilution culture methods. Plasma membranes were collected from homogenized HEK293 cells and used in the receptor binding assay. Individual test peptides at concentrations spanning 6 log units starting at around 10^{-6}M were examined for their ability to displace [^{125}I]-human calcitonin from the plasma membrane preparation using a 96-well microtiter plate format and scintillation counting with a Wallac LKB Beta plate counter. Competitive binding curves were constructed. Nonspecific binding was measured in the presence of 100 nM calcitonin. Peptide potencies (IC_{50}'s for competitive binding) were calculated by non-linear regression using Prism™ (version 2.01, GraphPAD Software, San Diego, Calif.). The results are reported in Table I.

Peptide compounds of the present invention may be evaluated for amylin agonist activity using the methods of measuring the rate of gastric emptying disclosed in, for example, Young et al., *Diabetologia*, 38(6):642–648 (1995). In a phenol red method, which is described in Example D below, conscious rats receive by gavage an a caloric gel containing methyl cellulose and a phenol red indicator. Twenty minutes after gavage, animals are anesthetized using halothane, the stomach exposed and clamped at the pyloric and lower esophageal sphincters, removed and opened into an alkaline solution. Stomach content may be derived from the intensity of the phenol red in the alkaline solution, measured by absorbance at a wavelength of 560 nm. In a tritiated glucose method, conscious rats are gavaged with tritiated glucose in water. The rats are gently restrained by the tail, the tip of which is anesthetized using lidocaine. Tritium in the plasma separated from tail blood is collected at various timepoints and detected in a beta counter. Test compounds are normally administered about one minute before gavage. The results are reported in Table II.

Preferably, compounds of the present invention exhibit activity in the nucleus accumbens receptor binding assay on the order of less than about 1 to 100 nM, and more preferably less than about 10 nM. In the gastric emptying assays, preferred compounds show ED_{50} values on the order of less than about 100 μg/rat, and more preferably less than about 10 μg/rat.

Formulation and Administration

Compositions useful in the invention may conveniently be provided in the form of formulations suitable for parenteral (including intravenous, intramuscular and subcutaneous) or nasal or oral administration or suitably encapsulated or otherwise prepared by art-known methods for oral administration. A suitable administration format may best be determined by a medical practitioner for each patient individually. Pharmaceutically acceptable carriers and their formulation are described in standard formulation treatises, e.g., *Remington's Pharmaceutical Sciences* by E. W. Martin. See also Wang, Y. J. and Hanson, M. A. "Parenteral Formulations of Proteins and Peptides: Stability and Stabilizers," *Journal of Parenteral Science and Technology*, Technical Report No. 10, Supp. 42:2S (1988).

Compounds useful in the invention can be provided as parenteral compositions for injection or infusion can, for example, be suspended in an inert oil, suitably a vegetable oil such as sesame, peanut, olive oil, or other acceptable carrier. Preferably, they are suspended in an aqueous carrier, for example, in an isotonic buffer solution at a pH of about 5.6 to 7.4. These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH buffering agents. Useful buffers include for example, sodium acetate/acetic acid buffers. A form of repository or "depot" slow release preparation may be used so that therapeutically effective amounts of the preparation are delivered into the bloodstream over many hours or days following transdermal injection or delivery.

Preferably, these parenteral dosage forms are prepared according to the commonly owned patent applications entitled, "Parenteral, Liquid Formulations for Amylin Agonist Peptides," Ser. No. 60/035,140, filed Jan. 8, 1997, and U.S. application Ser. No. 09/005,262, filed Jan. 8, 1998, which are incorporated herein by this reference, and include approximately 0.01 to 0.5% (w/v), respectively, of a compound in an aqueous system along with approximately 0.02 to 0.5% (w/v) of an acetate, phosphate, citrate or glutamate buffer to obtain a pH of the final composition of approximately 3.0 to 6.0 (more preferably 3.0 to 5.5), as well as approximately 1.0 to 10% (w/v) of a carbohydrate or polyhydric alcohol tonicifier in an aqueous continuous phase. Approximately 0.005 to 1.0% (w/v) of an antimicrobial preservative selected from the group consisting of m-cresol, benzyl alcohol, methyl, ethyl, propyl and butyl parabens and phenol is also present in the preferred formulation of product designed to allow the patient to withdraw multiple doses. A sufficient amount of water for injection is used to obtain the desired concentration of solution. Sodium chloride, as well as other excipients, may also be present, if desired. Such excipients, however, must maintain the overall stability of the peptide. Liquid formulations should be substantially

isotonic, that is, within ±20% of isotonicity, and preferably within 10% isotonicity. Most preferably, in the formulation for parenteral administration, the polyhydric alcohol is mannitol, the buffer is an acetate buffer, the preservative is approximately 0.1 to 0.3 w/v % of m-cresol, and the pH is approximately 3.7 to 4.3.

The desired isotonicity may be accomplished using sodium chloride or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol, polyols (such as mannitol and sorbitol), or other inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions. If desired, solutions of the above compositions may be thickened with a thickening agent such as methyl cellulose. They may be prepared in emulsified form, either water in oil or oil in water. Any of a wide variety of pharmaceutically acceptable emulsifying agents may be employed including, for example, acacia powder, a non-ionic surfactant (such as a Tween), or an ionic surfactant (such as alkali polyether alcohol sulfates or sulfonates, e.g., a Triton).

Compositions useful in the invention are prepared by mixing the ingredients following generally accepted procedures. For example, the selected components may be simply mixed in a blender or other standard device to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water or thickening agent and possibly a buffer to control pH or an additional solute to control tonicity.

For use by the physician, the compositions will be provided in dosage unit form containing an amount of a compound of the present invention, for example, a compound which will be effective in one or multiple doses to provide a therapeutic effect at the selected level. Therapeutically effective amounts of a compound of the present invention for use in the control of hyperglycemia, including hyperglycemia associated with insulin resistance, are those that significantly lower post-prandial glucose levels with respect to control, as may be measured by comparing the area under the curve of postprandial glucose concentrations. As will be recognized by those in the field, an effective amount of therapeutic agent will vary with many factors including the age and weight of the patient, the patient's physical condition, the action to be obtained and other factors.

The effective single, divided or continuous doses of the compounds will typically be in the range of about 0.1 μg/kg/day to about 1,000 μg/kg/day, preferably about 1.0 μg/kg/day to about 100 μg/kg/day, administered in a single dose or multiple doses.

As will be recognized by those in the field, an effective amount of therapeutic agent will vary with many factors including the age and weight of the patient, the patient's physical condition and other factors. Orally active compounds may be taken orally, however, dosages should be increased 5–10 fold, or should be increased (or decreased) in the ratio described earlier.

To assist in understanding the present invention, the following Examples are included which describe the results of several experiments. The experiments relating to this invention should not, of course, be construed as specifically limiting the invention and such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are considered to fall within the scope of the invention as described herein and hereinafter claimed.

EXAMPLES

Example 1

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Thr Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 1).

The above peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.). In general, single-coupling cycles were used throughout the synthesis and HATU chemistry (O-(7-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorphosphate) in the presence of diisopropylethylamine using N-methyl pyrrolidine as solvent was employed. However, at some positions coupling was less efficient than expected and double couplings were required. Deprotection (Fmoc group removal) of the growing peptide chain was achieved using piperidine. The N-terminus was completed using (bis-tBoc)-Lysine in the final coupling cycle. Final deprotection of the completed peptide resin was achieved using a mixture of triethylsilane (0.2 mL), ethanedithiol (0.2 mL), anisole (0.2 mL), water (0.2 mL) and trifluoroacetic acid (15 mL) according to standard methods (Introduction to Cleavage Techniques, Applied Biosystems, Inc.) The peptide was precipitated in ether/water (50 mL) and centrifuged. The precipitate was reconstituted in glacial acetic acid and lyophilized and the resulting crude peptide was redissolved in water and treated briefly with tris-carboethoxy phosphine to ensure complete generation of free thiols. Exposure to potassium ferricyanide at pH 6.5 effected cyclization to the mono-disulfide bridged peptide. Acidification and treatment with Biorad AG4X4 anion exchange resin removed any residual Fe^{2+} and Fe^{3+} ions. Lyophilisation gave the crude peptide. Crude purity was about 75%.

Used in purification steps and analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN).

The solution containing peptide was applied to a preparative C-18 column and purified (10% to 40% Solvent B in Solvent A over 40 minutes). Purity of fractions was determined isocratically using a C-18 analytical column. Pure fractions were pooled furnishing the above-identified peptide. Analytical RP-HPLC (gradient 5% to 95% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 12.96 minutes. Electrospray Mass Spectrometry (M): calculated 2272.12; found 2273.76 (M+H).

Example 2

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Val Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 2).

The above peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 60% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 18.48 minutes. Electrospray Mass Spectrometry (M): calculated 2398.2; found 2399.9 (M+H).

Example 3

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Cys Asn Thr Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser-NH_2 (SEQ. ID. NO. 3).

The above-identified peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. The sequence was completed after addition of the second protected cysteine residue. Used in analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 16.93 minutes. Electrospray Mass Spectrometry (M): calculated 1836.91; found 1838.9 (M+H).

Example 4

Preparation of Amidated Peptide Having the Sequence:

isocaproyl-Leu Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser-NH_2 (SEQ. ID. NO. 4).

The above peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.). In general, single-coupling cycles were used throughout the synthesis and HATU chemistry (O-(7-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorphosphate) in the presence of diisopropylethylamine using N-methyl pyrrolidine as solvent was employed. However, at some positions coupling was less efficient than expected and double couplings were required. Deprotection (Fmoc group removal) of the growing peptide chain was achieved using piperidine. The N-terminus was completed using isocaproyl-Leucine in the final coupling cycle. Final deprotection of the completed peptide resin was achieved using a mixture of triethylsilane (0.2 mL), ethanedithiol (0.2 mL), anisole (0.2 mL), water (0.2 mL) and trifluoroacetic acid (15 mL) according to standard methods (Introduction to Cleavage Techniques, Applied Biosystems, Inc.). The peptide was precipitated in ether/water (50 mL) and centrifuged. The precipitate was reconstituted in glacial acetic acid and lyophilized and the resulting crude peptide was redissolved in water. Lyophilisation gave the crude peptide. Crude purity was about 75%.

Used in purification steps and analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN).

The solution containing peptide was applied to a preparative C-18 column and purified (10% to 40% Solvent B in Solvent A over 40 minutes). Purity of fractions was determined isocratically using a C-18 analytical column. Pure fractions were pooled furnishing the above-identified peptide. Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 29.17 minutes. Electrospray Mass Spectrometry (M): calculated 1716.00; found 1716.85 (M+H).

Example 5

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Thr Ala Thr Cys Ala Thr Ala Arg Leu Ala Asn Phe Leu Val Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 5).

The above peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 20.57 minutes. Electrospray Mass Spectrometry (M): calculated 2343.16; found 2344.24 (M+H).

Example 6

Preparation of Amidated Peptide Having the Sequence:

Leu Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser-NH_2 (SEQ. ID. NO. 6).

The above peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.). In general, single-coupling cycles were used throughout the synthesis and HATU chemistry (O-(7-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorphosphate) in the presence of diisopropylethylamine using N-methyl pyrrolidine as solvent was employed. However, at some positions coupling was less efficient than expected and double couplings were required. Deprotection (Fmoc group removal) of the growing peptide chain was achieved using piperidine. Final deprotection of the completed peptide resin was achieved using a mixture of triethylsilane (0.2 mL), ethanedithiol (0.2 mL), anisole (0.2 mL), water (0.2 mL) and trifluoroacetic acid (15 mL) according to standard methods (Introduction to Cleavage Techniques, Applied Biosystems, Inc.). The peptide was precipitated in ether/water (50 mL) and centrifuged. The precipitate was reconstituted in glacial acetic acid and lyophilized and the resulting crude peptide was redissolved in water. Lyophilisation gave the crude peptide. Crude purity was about 75%.

Used in purification steps and analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN).

The solution containing peptide was applied to a preparative C-18 column and purified (10% to 40% Solvent B in Solvent A over 40 minutes). Purity of fractions was determined isocratically using a C-18 analytical column. Pure fractions were pooled furnishing the above-identified peptide. Analytical RP-HPLC (gradient 30% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 16.96 minutes. Electrospray Mass Spectrometry (M): calculated 1617.93; found 1618.73 (M+H).

Example 7

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Cys Asn Thr Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg-NH_2 (SEQ. ID. NO. 7).

The above peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. The sequence was completed after addition of the second protected cysteine residue. Used in analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 35% to 65% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 8.63 minutes.

Electrospray Mass Spectrometry (M): calculated 1749.88; found 1750.96 (M+H).

Example 8

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Ala Phe Leu Val Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 8).

The above-identified peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 20.76 minutes. Electrospray Mass Spectrometry (M): calculated 2357.17; found 2357.6 (M+H).

Example 9

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val Arg Ser Gly Tyr-NH_2 (SEQ. ID. NO. 9).

The above-identified peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 19.66 minutes. Electrospray Mass Spectrometry (M): calculated 2313.15; found 2314.77 (M+H).

Example 10

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val Arg Ala Ser Gly Tyr-NH_2 (SEQ. ID. NO. 10).

The above-identified peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 17.35 minutes. Electrospray Mass Spectrometry (M): calculated 2384.18; found 2385.63 (M+H).

Example 11

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val His Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 11).

The above-identified peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 21.06 minutes. Electrospray Mass Spectrometry (M): calculated 2381.14; found 2382.30 (M+H)

Example 12

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val Ala Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 12).

The above-identified peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 16.19 minutes. Electrospray Mass Spectrometry (M): calculated 2315.12; found 2315.94 (M+H).

Example 13

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 13).

The above-identified peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. The sequence was completed after addition of the second protected cysteine residue. Used in analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 21.02 minutes. Electrospray Mass Spectrometry (M): calculated 2272.08; found 2272.9 (M+H).

Example 14

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 14).

The above-identified peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 16.76 minutes. Electrospray Mass Spectrometry (M): calculated 2400.18; found 2400.13.

Example 15

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val Arg Ser Ala Gly Tyr-NH_2 (SEQ. ID. NO. 15).

The above-identified peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 18.70 minutes. Electrospray Mass Spectrometry (M): calculated 2384.18; found 2385.04 (M+H).

Example 16

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Ala Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 16).

The above-identified peptide was assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis were Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide gave product peptide having an observed retention time of 18.85 minutes. Electrospray Mass Spectrometry (M): calculated 2372.15; found 2372.97 (M+H).

Example 17

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 17).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2270.14.

Example 18

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 18).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2251.10.

Example 19

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Leu Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 19).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2284.16.

Example 20

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Leu Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 20).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2265.11.

Example 21

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Met Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 21).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide Adnorleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2302.11.

Example 22

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Met Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 22).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized

peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2283.07.

Example 23

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Leu Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 23).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2412.22.

Example 24

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Leu Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val His Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 24).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2393.17.

Example 25

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Asn Phe Leu Val Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 25).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2341.18.

Example 26

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Lys Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Asn Phe Leu Val His Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 26).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2322.13.

Example 27

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 27).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. The sequence is complete after addition of the second protected cysteine residue. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2142.05.

Example 28

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 28).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. The sequence is complete after addition of the second protected cysteine residue. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2123.00.

Example 29

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Cys Asn Leu Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 29).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. The sequence is complete after addition of the second protected cysteine residue. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2156.06.

Example 30

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Cys Asn Leu Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 30).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. The sequence is complete after addition of the second protected cysteine residue. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2137.02.

Example 31

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Cys Asn Met Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 31).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. The sequence is complete after addition of the second protected cysteine residue. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2174.02.

Example 32

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Cys Asn Met Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 32).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. The sequence is complete after addition of the second protected cysteine residue. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2154.98.

Example 33

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Cys Asn Leu Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 33).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. The sequence is complete after addition of the second protected cysteine residue. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2284.12.

Example 34

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Cys Asn Leu Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val His Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 34).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. The sequence is complete after addition of the second protected cysteine residue. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2265.08.

Example 35

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Asn Phe Leu Val Arg Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 35).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. The sequence is complete after addition of the second protected cysteine residue. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2213.08.

Example 36

Preparation of Mono-disulfide Bridged Amidated Peptide Having the Sequence:

Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Asn Phe Leu Val His Ser Ser Gly Tyr-NH_2 (SEQ. ID. NO. 36).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected, cyclized and purified in a similar way to Example 1. The sequence is complete after addition of the second protected cysteine residue. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 2194.03.

Example 37

Preparation of Amidated Peptide Having the Sequence: isocaproyl-Leu Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser-NH_2 (SEQ. ID. NO. 37).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected and purified in a similar way to Example 4. The N-terminus is completed using isocaproyl-Leucine in the final coupling cycle. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 1695.96.

Example 38

Preparation of Amidated Peptide Having the Sequence: isocaproyl-Val Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser-NH_2 (SEQ. ID. NO. 38).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected and purified in a similar way to Example 4. The N-terminus is completed using isocaproyl-Valine in the final coupling cycle. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 1700.98.

Example 39

Preparation of Amidated Peptide Having the Sequence: isocaproyl-Val Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser-NH_2 (SEQ. ID. NO. 39).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected and purified in a similar way to Example 4. The N-terminus is completed using isocaproyl-Valine in the final coupling cycle. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 1681.94.

Example 40

Preparation of Amidated Peptide Having the Sequence: isocaproyl-Met Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser-NH_2 (SEQ. ID. NO. 40).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected and purified in a similar way to Example 4. The N-terminus is completed using isocaproyl-Methionine in the final coupling cycle. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 1732.95.

Example 41

Preparation of Amidated Peptide Having the Sequence: isocaproyl-Met Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser-NH_2 (SEQ. ID. NO. 41).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected and purified in a similar way to Example 4. The N-terminus is completed using isocaproyl-Methionine in the final coupling cycle. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 1713.91.

Example 42

Preparation of Amidated Peptide Having the Sequence: Leu Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser-NH_2 (SEQ. ID. NO. 42).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected and purified in a similar way to Example 6. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 1598.89.

Example 43

Preparation of Amidated Peptide Having the Sequence: Val Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser-NH_2 (SEQ. ID. NO. 43).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected and purified in a similar way to Example 6. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 1603.92.

Example 44

Preparation of Amidated Peptide Having the Sequence: Val Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser-NH_2 (SEQ. ID. NO. 44).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected and purified in a similar way to Example 6. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in

Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 1584.87.

Example 45

Preparation of Amidated Peptide Having the Sequence: Met Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser-NH_2 (SEQ. ID. NO. 45).

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected and purified in a similar way to Example 6. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 1635.89.

Example 46

Preparation of Amidated Peptide Having the Sequence: Met Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser-NH_2 (SEQ. ID. NO. 46)

The above-identified peptide is assembled on 4-(2'-4'-dimethoxyphenyl)-Fmoc aminomethyl phenoxy acetamide norleucine MBHA resin (Novabiochem, 0.55 mmole/g) using Fmoc-protected amino acids (Applied Biosystems, Inc.), cleaved from the resin, deprotected and purified in a similar way to Example 6. Used in analysis are Solvent A (0.1% TFA in water) and Solvent B (0.1% TFA in CH_3CN). Analytical RP-HPLC (gradient 20% to 50% Solvent B in Solvent A over 30 minutes) of the lyophilized peptide is then carried out to determine the retention time of the product peptide. Electrospray Mass Spectrometry (M): calculated 1616.85.

Example A

Binding to Rat Nucleus Accumbens Membranes (Amylin Receptors)

Evaluation of the binding of compounds to amylin receptors was carried out as follows. ^{125}I-BH-rat amylin was purchased from Amersham Corporation (Arlington Heights, Ill.). Specific activities at time of use ranged from 1950 to 2000 Ci/mmol. Unlabeled reference peptides were obtained from BACHEM Inc. (Torrance, Calif.) and Penninsula Laboratories (Belmont, Calif.).

Male Sprague-Dawley rats (200 to 250 grams) were sacrificed by decapitation. Brains were removed to cold phosphate-buffered saline (PBS). From the ventral surface, cuts were made rostral to the hypothalamus, bounded laterally by the olfactory tracts and extending at a 45°angle medially from these tracts. This basal forebrain tissue, containing the nucleus accumbens and surrounding regions, was weighed and homogenized in ice-cold 20 mM HEPES buffer (20 mM HEPES acid, pH adjusted to 7.4 with NaOH at 23° C.). Membranes were washed three times in fresh buffer by centrifugation for 15 minutes at 48,000×g. The final membrane pellet was resuspended in 20 mM HEPES buffer containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF).

To measure ^{125}I-amylin binding, membranes from 4 mg original wet weight of tissue were incubated with ^{125}I-amylin at 12 to 16 pM in 20 mM HEPES buffer containing 0.5 mg/mL bacitracin, 0.5 mg/mL bovine serum albumin, and 0.2 mM PMSF. Solutions were incubated for 60 minutes at 23° C. Incubations were terminated by filtration through GF/B glass fiber filters (Whatman Inc., Clifton, N.J.) which had been presoaked for 4 hours in 0.3% poylethyleneimine in order to reduce nonspecific binding of radiolabeled peptides. Filters were washed immediately before filtration with 5 mL cold PBS, and immediately after filtration with 15 mL cold PBS. Filters were removed and radioactivity assessed in a gamma-counter at a counting efficiency of 77%.

Results, reported as IC_{50}'s,are set forth in Table I.

Example B

[^{35}S]-GTPγS C1a Agonist Studies

Evaluation of the agonist activity of compounds versus the C1a receptor was carried out as follows:

Reference peptides used in these studies were purchased from Bachem (Torrance, Calif.). All other chemicals were of the highest commercial grade. [^{35}S]-GTPγS was purchased from NEN Life Science Products, Inc., Pittsburgh, Pa. [^{125}I]-human calcitonin was purchased from Amersham Pharmacia Biotech, Inc., Piscataway, N.J.

Methods for C1a pcDNA construction and transfection have been previously described (Albrandt, et al. 1993, FEBS Letters, 325:225–232). HEK293 cell lines stably expressing the rat C1a-type calcitonin receptor (C1a/293) or the human C1a-type calcitonin receptor (1154/293) were selected by G418 resistance and limiting dilution culture methods.

Confluent cells were detached from tissue culture flasks by incubation with 5 mM EDTA in PBS. Cells were homogenized in ice-cold 20 mM HEPES, pH 7.4 with a Polytron homogenizer. Plasma membranes were collected using three cycles of washing in fresh buffer followed by centrifugation for 15 minutes at 48,000×g. The final membrane pellet was resuspended in 20 mM HEPES buffer containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and stored at −70° C.

Assay buffer contained 1 μM GDP, 1 mM EDTA, 5 mM $MgCl_2$, 20 mM Hepes, and 150 mM NaCl, pH 7.4. Membranes (7.5 μg membrane protein/well), [^{35}S]-GTPγS (200 pM), and peptides were combined in 200 μL buffer in 96-well microtiter plates. After a 75 minute incubation at room temperature, well contents were harvested onto GF/B glass fiber pads using a Tomtec Mach II plate harvestor (Hamden, Conn.). Dried pads were combined with scintillant and counted on a Wallac LKB Beta Plate scintillation counter. For concentration response curves, samples were run in duplicate over a 6 log concentration range starting at 10^{-6} M or 10^{-7} M. Maximum agonist-specific binding was measured in the presence of 1 μM human calcitonin, constitutive binding was measured in the presence of buffer alone.

Peptide potencies ($1C_{50}$'s for competitive binding, and EC_{50}'s for concentration response curves) were calculated by non-linear regression using Prism (version 2.01, GraphPAD Software, San Diego, Calif.).

Results, reported as ED_{50}'s, are set forth in Table I.

Example C

C1a/293 Competitive Binding Studies:

Compounds were evaluated for competition in binding to the C1a receptor as follows:

Reference peptides used in these studies were purchased from Bachem (Torrance, Calif.). All other chemicals were of the highest commercial grade. [^{35}S]-GTPγS was purchased from NEN Life Science Products, Inc., Pittsburgh, Pa. [^{125}I]-human calcitonin was purchased from Amersham Pharmacia Biotech, Inc., Piscataway, N.J.

Methods for C1a pcDNA construction and transfection have been previously described (Albrandt, et al. 1993). HEK293 cell lines stably expressing the rat C1a-type cal-

citonin receptor (C1a/293) or the human C1a-type calcitonin receptor (1154/293) were selected by G418 resistance and limiting dilution culture methods.

Confluent cells were detached from tissue culture flasks by incubation with 5 mM EDTA in PBS. Cells were homogenized in ice-cold 20 mM HEPES, pH 7.4 with a Polytron homogenizer. Plasma membranes were collected using three cycles of washing in fresh buffer followed by centrifugation for 15 minutes at 48,000×g. The final membrane pellet was resuspended in 20 mM HEPES buffer containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and stored at −70° C.

Assay buffer contained 5 μg/mL bestatin, 1 μg/mL phosphoramidon, 1 mg/mL bovine serum albumin (fraction V), 1 mg/mL bacitracin, 1 mM $MgCl_2$, and 20 mM HEPES, pH 7.4. Membranes (2–5 μg membrane protein/well), [^{125}I]-human calcitonin (20 pM), and peptides were combined in 200 μL buffer in 96-well microtiter plates. After a 60 minute incubation at room temperature, well contents were harvested onto polyethyleneimine-treated GF/B glass fiber pads using a Tomtec Mach II plate harvestor (Hamden, Conn.). Dried pads were combined with scintillant and counted on a Wallac LKB Beta Plate scintillation counter (Gaithersburg, Md.). For competitive binding curves, samples were run in duplicate over a 6 log concentration range starting at 10^{-6} or 10^{-7} M. Non-specific binding was measured in the presence of 100 nM human calcitonin.

Results, reported as IC_{50}'s, are set forth in Table I.

TABLE I

		IC_{50} (nM)		ED_{50} (nM)
Example No.	SEQ. ID. NO.	Example A (rAmy)	Example C (C1a)	Example B (C1a GTPγS)
1	1	1.9	0.093	0.7
2	2	44.3	0.128	2.6
3	3	5.7	0.190	1.2
4	4	32	0.66	0.73
5	5	14	1.4	5.5
6	6	78	1.4	0.58
7	7	31	2.7	2.2
8	8	101	3.4	4.4
9	9	91	4.1	8.6
10	10	68	4.7	8
11	11	148	8.3	14
12	12	286	10	8
13	13	95	12	19
14	14	132	18	90
15	15	206	18	21
16	16	31	20	1.4

Example D

Phenol Red Gastric Emptying Assay:

Gastric emptying was measured using a modification (Plourde et al., Life Sci. 53:857–862 (1993)) of the original method of Scarpignato et al. (Arch. Int. Pharmacodyn. Ther. 246:286–295 (1980)). Conscious rats received by gavage. 1.5 mL of an a caloric gel containing 1.5% methyl cellulose (M-0262, Sigma Chemical Co., St. Louis, Mo.) and 0.05% phenol red indicator. Twenty minutes after gavage, rats were anesthetized using 5% halothane, the stomach exposed and clamped at the pyloric and lower esophageal sphincters using artery forceps, removed and opened into an alkaline solution which was made up to a fixed volume. Stomach content was derived from the intensity of the phenol red in the alkaline solution, measured by absorbance at a wavelength of 560 nm. In most experiments, the stomach was clear. In other experiments, particulate gastric contents were centrifuged to clear the solution for absorbance measurements. Where the diluted gastric contents remained turbid, the spectroscopic absorbance due to phenol red was derived as the difference between that present in alkaline versus acidified dilutent.

In separate experiments on 7 rats, the stomach and small intestine were both excised and opened into an alkaline solution. The quantity of phenol red that could be recovered from the upper gastrointestinal tract within 29 minutes of gavage was 89±4%; dye which appeared to bind irrecoverably to the gut luminal surface may have accounted for the balance. To compensate for this small loss, percent of stomach contents remaining after 20 minutes were expressed as a fraction of the gastric contents recovered from control rats sacrificed immediately after gavage in the same experiment. Percent gastric emptying contents remaining=(absorbance at 20 min)/(absorbance at 0 min). For those compounds for which ED_{50} data is presented, dose response curves for gastric emptying were fitted to a 4-parameter logistic model using a least-squares iterative routine (ALLFIT, v2.7, NIH, Bethesda, Md.) to derive ED_{50}s. Because ED_{50} is log-normally distributed, it is expressed ± standard error of the logarithm. Pairwise comparisons were performed using one-way analysis of variance and the Student-Newman-Keuls multiple comparisons test (Instat v2.0, GraphPad Software, San Diego, Calif.) using $P<0.05$ as the level of significance.

As a reference point for dose response studies, rat amylin (Bachem, Torrance, Calif.) dissolved in 0.15M saline, was administered as a 0.1 mL subcutaneous bolus in doses of 0, 0.01, 0.1, 1, 10 or 100 μg 5 minutes before gavage in Harlan Sprague Dawley (non-diabetic) rats fasted 20 hours and diabetic BB rats fasted 6 hours. When subcutaneous amylin injections were given 5 minutes before gavage with phenol red indicator, there was a dose-dependent suppression of gastric emptying (data not shown). Suppression of gastric emptying was complete in normal HSD rats administered 1 μg of amylin, and in diabetic rats administered 10 μg (P=0.22, 0.14). The ED_{50} for inhibition of gastric emptying in normal rats was 0.43 μg (0.60 nmol/kg) ±0.19 log units, and was 2.2 μg (2.3 nmol/kg) ±0.18 log units in diabetic rats.

Amylin (rat or human) and compounds that exhibit amylin-like actions in isolated soleus muscle (including, salmon calcitonin, CGRP, and rat calcitonin) have been observed to dose-dependently inhibit gastric emptying in the present conscious rat model. Adrenomedullin, which has been observed to behave as a CGRP agonist but not as an amylin or calcitonin agonist, does not inhibit gastric emptying at the highest doses (100 μg) used in this model (indicating that inhibition of gastric emptying in this model is unlikely to be mediated by CGRP receptors).

Results are set forth in Table II.

TABLE II

Example No.	SEQ. ID. NO.	Example D ED_{50} (μg) or % Remaining (100 μg)
1	1	* 0.26
3	3	* 0.45
5	5	* 9.3
6	6	* 1.34
13	13	69%
14	14	73%
rat amylin	N/A	* 0.26

*ED_{50}

SEQUENCE LISTING

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<220> FEATURE:
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<222> LOCATION: (2)...(7)
<223> OTHER INFORMATION: disulfide linkage between amino acid Cys at
      positions 2 and 7.
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (22)...(22)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

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Lys Cys Asn Thr Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu
 1              5                   10                  15

Ala Arg Ser Ser Gly Tyr
            20


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<223> OTHER INFORMATION: Synthesized amylin agonist
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<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 2

Lys Cys Asn Val Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu
 1              5                   10                  15

Val Arg Ser Ser Gly Tyr
            20


<210> SEQ ID NO 3
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<220> FEATURE:
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<223> OTHER INFORMATION: amidated Ser (serinamide)

<400> SEQUENCE: 3

Cys Asn Thr Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala
 1              5                   10                  15

Arg Ser
```

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<210> SEQ ID NO 4
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<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (17)...(17)
<223> OTHER INFORMATION: amidated Ser (serinamide)

<400> SEQUENCE: 4

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Xaa Leu Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg
 1               5                  10                  15

Ser
```

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<223> OTHER INFORMATION: disulfide linkage between amino acid Cys at
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<220> FEATURE:
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<222> LOCATION: (22)...(22)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 5

```
Lys Cys Asn Thr Ala Thr Cys Ala Thr Ala Arg Leu Ala Asn Phe Leu
 1               5                  10                  15

Val Arg Ser Ser Gly Tyr
            20
```

<210> SEQ ID NO 6
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<223> OTHER INFORMATION: amidated Ser (serinamide)

<400> SEQUENCE: 6

```
Leu Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser
 1               5                  10                  15
```

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<223> OTHER INFORMATION: disulfide linkage between amino acid Cys at
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<220> FEATURE:
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<222> LOCATION: (17)...(17)

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<223> OTHER INFORMATION: amidated Arg (argininamide)

<400> SEQUENCE: 7

Cys Asn Thr Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala
1 5 10 15

Arg

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<220> FEATURE:
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<222> LOCATION: (22)...(22)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 8

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Ala Phe Leu
1 5 10 15

Val Arg Ser Ser Gly Tyr
20

<210> SEQ ID NO 9
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<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (21)...(21)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 9

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu
1 5 10 15

Val Arg Ser Gly Tyr
20

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<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (22)...(22)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 10

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu

-continued

```
 1               5                   10                  15

Val Arg Ala Ser Gly Tyr
            20


<210> SEQ ID NO 11
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<220> FEATURE:
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<222> LOCATION: (22)...(22)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 11

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu
 1               5                   10                  15

Val His Ser Ser Gly Tyr
            20


<210> SEQ ID NO 12
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<220> FEATURE:
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<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 12

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu
 1               5                   10                  15

Val Ala Ser Ser Gly Tyr
            20


<210> SEQ ID NO 13
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<220> FEATURE:
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<222> LOCATION: (21)...(21)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 13

Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val
 1               5                   10                  15

Arg Ser Ser Gly Tyr
            20
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<210> SEQ ID NO 14
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<222> LOCATION: (22)...(22)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 14

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Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu
 1               5                  10                  15

Val Arg Ser Ser Gly Tyr
            20
```

<210> SEQ ID NO 15
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<220> FEATURE:
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<222> LOCATION: (22)...(22)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 15

```
Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu
 1               5                  10                  15

Val Arg Ser Ala Gly Tyr
            20
```

<210> SEQ ID NO 16
<211> LENGTH: 22
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<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 16

```
Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu
 1               5                  10                  15

Ala Arg Ser Ser Gly Tyr
            20
```

<210> SEQ ID NO 17
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<212> TYPE: PRT
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<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 17

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Lys Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu
 1               5                  10                  15

Ala Arg Ser Ser Gly Tyr
            20
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<210> SEQ ID NO 18
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<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 18

```
Lys Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu
 1               5                  10                  15

Ala His Ser Ser Gly Tyr
            20
```

<210> SEQ ID NO 19
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<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 19

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 1               5                  10                  15

Ala Arg Ser Ser Gly Tyr
            20
```

<210> SEQ ID NO 20
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<220> FEATURE:
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<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 20

Lys Cys Asn Leu Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu
1 5 10 15

Ala His Ser Ser Gly Tyr
20

<210> SEQ ID NO 21
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<220> FEATURE:
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<222> LOCATION: (22)...(22)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 21

Lys Cys Asn Met Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu
1 5 10 15

Ala Arg Ser Ser Gly Tyr
20

<210> SEQ ID NO 22
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<220> FEATURE:
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<222> LOCATION: (22)...(22)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 22

Lys Cys Asn Met Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu
1 5 10 15

Ala His Ser Ser Gly Tyr
20

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 1               5                  10                  15

Val Arg Ser Ser Gly Tyr
            20
```

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<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 24

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 1               5                  10                  15

Val His Ser Ser Gly Tyr
            20
```

<210> SEQ ID NO 25
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<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (22)...(22)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 25

```
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 1               5                  10                  15

Val Arg Ser Ser Gly Tyr
            20
```

<210> SEQ ID NO 26
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<223> OTHER INFORMATION: disulfide linkage between amino acid Cys at positions 2 and 7.
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<221> NAME/KEY: AMIDATION
<222> LOCATION: (22)...(22)

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<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 26

Lys Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Asn Phe Leu
1 5 10 15

Val His Ser Ser Gly Tyr
20

<210> SEQ ID NO 27
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<212> TYPE: PRT
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<223> OTHER INFORMATION: disulfide linkage between amino acid Cys at positions 1 and 6.
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (21)...(21)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 27

Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala
1 5 10 15

Arg Ser Ser Gly Tyr
20

<210> SEQ ID NO 28
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<212> TYPE: PRT
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<220> FEATURE:
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<222> LOCATION: (21)...(21)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 28

Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala
1 5 10 15

His Ser Ser Gly Tyr
20

<210> SEQ ID NO 29
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: DISULFID
<222> LOCATION: (1)...(6)
<223> OTHER INFORMATION: disulfide linkage between amino acid Cys at positions 1 and 6.
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (21)...(21)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 29

-continued

```
Cys Asn Leu Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala
 1               5                  10                  15

Arg Ser Ser Gly Tyr
            20


<210> SEQ ID NO 30
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: DISULFID
<222> LOCATION: (1)...(6)
<223> OTHER INFORMATION: disulfide linkage between amino acid Cys at
      positions 1 and 6.
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (21)...(21)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 30

Cys Asn Leu Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala
 1               5                  10                  15

His Ser Ser Gly Tyr
            20


<210> SEQ ID NO 31
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: DISULFID
<222> LOCATION: (1)...(6)
<223> OTHER INFORMATION: disulfide linkage between amino acid Cys at
      positions 1 and 6.
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (21)...(21)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 31

Cys Asn Met Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala
 1               5                  10                  15

Arg Ser Ser Gly Tyr
            20


<210> SEQ ID NO 32
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: DISULFID
<222> LOCATION: (1)...(6)
<223> OTHER INFORMATION: disulfide linkage between amino acid Cys at
      positions 1 and 6.
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (21)...(21)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 32

Cys Asn Met Ala Thr Cys Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala
 1               5                  10                  15

His Ser Ser Gly Tyr
```

-continued

```
            20


<210> SEQ ID NO 33
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: DISULFID
<222> LOCATION: (1)...(6)
<223> OTHER INFORMATION: disulfide linkage between amino acid Cys at
      positions 1 and 6.
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (21)...(21)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 33

Cys Asn Leu Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val
 1               5                  10                  15

Arg Ser Ser Gly Tyr
            20


<210> SEQ ID NO 34
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: DISULFID
<222> LOCATION: (1)...(6)
<223> OTHER INFORMATION: disulfide linkage between amino acid Cys at
      positions 1 and 6.
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (21)...(21)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 34

Cys Asn Leu Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu Val
 1               5                  10                  15

His Ser Ser Gly Tyr
            20


<210> SEQ ID NO 35
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: DISULFID
<222> LOCATION: (1)...(6)
<223> OTHER INFORMATION: disulfide linkage between amino acid Cys at
      positions 1 and 6.
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (21)...(21)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 35

Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Asn Phe Leu Val
 1               5                  10                  15

Arg Ser Ser Gly Tyr
            20


<210> SEQ ID NO 36
```

-continued

```
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: DISULFID
<222> LOCATION: (1)...(6)
<223> OTHER INFORMATION: disulfide linkage between amino acid Cys at
      positions 1 and 6.
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (21)...(21)
<223> OTHER INFORMATION: amidated Tyr (tyrosinamide)

<400> SEQUENCE: 36

Cys Asn Val Ala Thr Cys Ala Thr Ala Arg Leu Ala Asn Phe Leu Val
 1               5                  10                  15

His Ser Ser Gly Tyr
            20


<210> SEQ ID NO 37
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: SITE
<222> LOCATION: (1)...(1)
<223> OTHER INFORMATION: Xaa in position 1 stands for isocaproyl-Leu
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (17)...(17)
<223> OTHER INFORMATION: amidated Ser (serinamide)

<400> SEQUENCE: 37

Xaa Leu Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His
 1               5                  10                  15

Ser


<210> SEQ ID NO 38
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: SITE
<222> LOCATION: (1)...(1)
<223> OTHER INFORMATION: Xaa in position 1 stands for isocaproyl-Val
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (17)...(17)
<223> OTHER INFORMATION: amidated Ser (serinamide)

<400> SEQUENCE: 38

Xaa Val Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg
 1               5                  10                  15

Ser


<210> SEQ ID NO 39
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: SITE
<222> LOCATION: (1)...(1)
```

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<223> OTHER INFORMATION: Xaa in position 1 stands for isocaproyl-Val
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (17)...(17)
<223> OTHER INFORMATION: amidated Ser (serinamide)

<400> SEQUENCE: 39

```
Xaa Val Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His
 1               5                  10                  15

Ser
```

<210> SEQ ID NO 40
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: SITE
<222> LOCATION: (1)...(1)
<223> OTHER INFORMATION: Xaa in position 1 stands for isocaproyl-Met
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (17)...(17)
<223> OTHER INFORMATION: amidated Ser (serinamide)

<400> SEQUENCE: 40

```
Xaa Met Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg
 1               5                  10                  15

Ser
```

<210> SEQ ID NO 41
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: SITE
<222> LOCATION: (1)...(1)
<223> OTHER INFORMATION: Xaa in position 1 stands for isocaproyl-Met
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (17)...(17)
<223> OTHER INFORMATION: amidated Ser (serinamide)

<400> SEQUENCE: 41

```
Xaa Met Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His
 1               5                  10                  15

Ser
```

<210> SEQ ID NO 42
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (16)...(16)
<223> OTHER INFORMATION: amidated Ser (serinamide)

<400> SEQUENCE: 42

```
Leu Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser
 1               5                  10                  15
```

<210> SEQ ID NO 43
<211> LENGTH: 16

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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (16)...(16)
<223> OTHER INFORMATION: amidated Ser (serinamide)

<400> SEQUENCE: 43

Val Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser
1 5 10 15

<210> SEQ ID NO 44
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (16)...(16)
<223> OTHER INFORMATION: amidated Ser (serinamide)

<400> SEQUENCE: 44

Val Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser
1 5 10 15

<210> SEQ ID NO 45
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (16)...(16)
<223> OTHER INFORMATION: amidated Ser (serinamide)

<400> SEQUENCE: 45

Met Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala Arg Ser
1 5 10 15

<210> SEQ ID NO 46
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amylin agonist
<220> FEATURE:
<221> NAME/KEY: AMIDATION
<222> LOCATION: (16)...(16)
<223> OTHER INFORMATION: amidated Ser (serinamide)

<400> SEQUENCE: 46

Met Ser Thr Ala Ala Thr Ala Arg Leu Ala Ala Phe Leu Ala His Ser
1 5 10 15

<210> SEQ ID NO 47
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthesized amino acid
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (1)...(4)
<223> OTHER INFORMATION: Xaa in position 1 is Asn, Gln or Asp; Xaa in position 2 is Thr, Ser, Met, Val, Leu or Ile; Xaa in position 3 is Ala or Val; Xaa in position 4 is Thr or Ser.

-continued

<400> SEQUENCE: 47

Xaa Xaa Xaa Xaa
1

<210> SEQ ID NO 48
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 48

Arg Ser Ser Gly Tyr
1 5

<210> SEQ ID NO 49
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 49

Lys Ser Ser Gly Tyr
1 5

<210> SEQ ID NO 50
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 50

His Ser Ser Gly Tyr
1 5

<210> SEQ ID NO 51
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 51

Pro Ser Ser Gly Tyr
1 5

<210> SEQ ID NO 52
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 52

Arg Ser Arg Gly Tyr
1 5

<210> SEQ ID NO 53
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 53

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Arg Thr Ser Gly Tyr
1 5

<210> SEQ ID NO 54
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 54

Arg Ala Ser Gly Tyr
1 5

<210> SEQ ID NO 55
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 55

Ala Ser Ser Gly Tyr
1 5

<210> SEQ ID NO 56
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 56

Arg Ser Ala Gly Tyr
1 5

<210> SEQ ID NO 57
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 57

His Ser Ala Gly Tyr
1 5

<210> SEQ ID NO 58
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 58

Arg Ser Gly Tyr
1

<210> SEQ ID NO 59
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 59

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```
Asn Thr Ala Thr
 1


<210> SEQ ID NO 60
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 60

Asn Val Ala Thr
 1


<210> SEQ ID NO 61
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 61

Asn Leu Ala Thr
 1


<210> SEQ ID NO 62
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 62

Asn Met Ala Thr
```

We claim:

1. A compound of the formula:

X_1-Xaa_1-X_2-Xaa_2-X_3-Xaa_3-X_4-Xaa_4-X_5-Xaa_5-X_6-NH_2 wherein X_1 is Lys, Arg or absent;

X_2 is $Xaa_6Xaa_7Xaa_8Xaa_9$ (SEQ. ID. NO. 47) or Z-Xaa_{10}SerThr, provided that if X_2 is Z-Xaa_{10}Ser-Thr, then X_1 and Xaa_1 are both absent;

X_3 is AlaThr, AlaSer, SerMet, GluThr or ValThr;

X_4 is ArgLeuAla, HisLeuAla, ArgIleAla, LysIleAla, ArgMetAla, HisMetAla, LysMetAla or ArgLeuThr;

X_5 is PheLeu, PheIle, PheMet, TyrLeu, TyrIle, TyrMet, TrpMet, TrpIle or TrpMet;

X_6 is ArgSerSerGlyTyr (SEQ. ID. NO. 48), LysSerSerGlyTyr (SEQ. ID. NO. 49), HisSerSerGlyTyr (SEQ. ID. NO. 50), ProSerSerGlyTyr (SEQ. ID. NO. 51), ArgSerArgGlyTyr (SEQ. ID. NO. 52), ArgThrSerGlyTyr (SEQ. ID. NO. 53), ArgAlaSerGlyTyr (SEQ. ID. NO. 54), AlaSerSerGlyTyr (SEQ. ID. NO. 55), ArgSerAlaGlyTyr (SEQ. ID. NO. 56), HisSerAlaGlyTyr (SEQ. ID. NO. 57), ArgSerGlyTyr (SEQ. ID. NO. 58), ArgSer, LysSer, HisSer, ArgThr, ProSer or Arg;

Xaa_1 is Cys or absent;

Xaa_2 is Cys or Ala;

Xaa_3 is Gln, Ala or Asn;

Xaa_4 is Asn, Ala or Gln;

Xaa_5 is Val, Ala, Ile, Met, Leu, PentylGly, or t-butylGly;

Xaa_6 is Asn, Gln or Asp;

Xaa_7 is Thr, Ser, Met, Val, Leu or Ile;

Xaa_8 is Ala or Val;

Xaa_9 is Thr or Ser;

Xaa_{10} is Leu, Val, Met or Ile; and

Z is an alkanoyl group of about 1 to about 8 carbon atoms or absent and pharmaceutically acceptable salts thereof.

2. A compound according to claim 1 wherein X_3 is AlaThr.

3. A compound according to claim 2 wherein X_4 is ArgLeuAla.

4. A compound according to claim 3 wherein X_5 is PheLeu.

5. A compound according to claim 4 wherein X_6 is ArgSerSerGlyTyr (SEQ. ID. NO. 48), HisSerSerGlyTyr (SEQ. ID. NO. 50), ArgSer or HisSer.

6. A compound according to claim 5 wherein X_6 is ArgSerSerGlyTyr (SEQ. ID. NO. 48) or ArgSer.

7. A compound according to claim 5 wherein X_6 is ArgSerSerGlyTyr (SEQ. ID. NO. 48) or HisSerSerGlyTyr (SEQ. ID. NO. 50).

8. A compound according to claim 7 wherein Xaa_1 is Cys.

9. A compound according to claim 8 wherein Xaa_2 is Cys.

10. A compound according to claim 9 wherein Xaa_1 and Xaa_2 form a disulfide bridge.

11. A compound according to claim 9 wherein X_2 is AsnThrAlaThr (SEQ. ID. NO. 59), AsnValAlaThr (SEQ. ID. NO. 60), AsnLeuAlaThr (SEQ. ID. NO. 61), or AsnMetAlaThr (SEQ. ID. NO. 62).

12. A compound according to claim 11 wherein Xaa_3 is Ala or Gln.

13. A compound according to claim 12 wherein Xaa_4 is Ala or Asn.

14. A compound according to claim **13** wherein Xaa_5 is Ala or Val.

15. A compound according to claim **14** wherein X_2 is AsnThrAlaThr (SEQ. ID. NO. 59) or AsnValAlaThr (SEQ. ID. NO. 60).

16. A compound according to claim **15** wherein Xaa_1 and Xaa_2 form a disulfide bridge.

17. A compound according to claim **15** wherein X_6 is ArgSerSerGlyTyr (SEQ. ID. NO. 48).

18. A compound according to claim **17** wherein X_2 is AsnThrAlaThr (SEQ. ID. NO. 59).

19. A compound according to claim **18** wherein X_1 is Lys.

20. A compound according to claim **19** wherein Xaa_3 is Ala.

21. A compound according to claim **20** wherein Xaa_4 is Ala.

22. A compound according to claim **21** wherein Xaa_5 is Ala.

23. A compound according to claim **22** wherein Xaa_1 and Xaa_2 form a disulfide bridge.

24. A compound according to claim **12** wherein X_2 is AsnValAlaThr (SEQ. ID. NO. 60).

25. A compound according to claim **24** wherein Xaa_3 is Gln.

26. A compound according to claim **25** wherein Xaa_4 is Asn.

27. A compound according to claim **26** wherein Xaa_5 is Val.

28. A compound according to claim **27** wherein X_1 is absent.

29. A compound according to claim **28** wherein Xaa_1 and Xaa_2 form a disulfide bridge.

30. A compound according to claim **5** wherein X_6 is ArgSer or HisSer.

31. A compound according to claim **30** wherein Xaa_1 is Cys.

32. A compound according to claim **31** wherein Xaa_2 is Cys.

33. A compound according to claim **32** wherein Xaa_1 and Xaa_2 form a disulfide bridge.

34. A compound according to claim **32** wherein X_2 is AsnThrAlaThr (SEQ. ID. NO. 59) or AsnValAlaThr (SEQ. ID. NO. 60).

35. A compound according to claim **34** wherein X_6 is ArgSer.

36. A compound according to claim **35** wherein X_2 is AsnThrAlaThr (SEQ. ID. NO. 59).

37. A compound according to claim **36** wherein Xaa_3 is Ala.

38. A compound according to claim **37** wherein Xaa_4 is Ala.

39. A compound according to claim **38** wherein Xaa_5 is Ala.

40. A compound according to claim **39** wherein Xaa_1 and Xaa_2 form a disulfide bridge.

41. A compound according to claim **5** wherein X_1 is absent.

42. A compound according to claim **41** wherein Xaa_1 is absent.

43. A compound according to claim **42** wherein X_6 is ArgSer or HisSer.

44. A compound according to claim **43** wherein X_2 is Z-Xaa_{10}SerThr.

45. A compound according to claim **44** wherein Xaa_{10} is Leu Val or Met.

46. A compound according to claim **45** wherein Xaa_4 is Ala.

47. A compound according to claim **46** wherein Xaa_3 is Ala.

48. A compound according to claim **47** wherein Xaa_5 is Ala.

49. A compound according to claim **48** wherein X_6 is ArgSer.

50. A compound according to claim **49** wherein Xaa_{10} is Leu.

51. A compound according to claim **1** wherein X_1 is absent.

52. A compound according to claim **51** wherein Xaa_1 is absent.

53. A compound according to claim **52** wherein X_2 is Z-Xaa_{10}SerThr.

54. A compound according to claim **1** wherein X_2 is $Xaa_6Xaa_7Xaa_8Xaa_9$ (SEQ. ID. NO. 47).

55. A compound according to claim **54** wherein Xaa_6 is Asn, Xaa_8 is Ala and Xaa_9 is Thr.

56. A compound according to claim **1** having an amino acid sequence selected from SEQ. ID. NOS. 1 to 46.

57. A compound according to claim **56** having an amino acid sequence selected from SEQ. ID. NOS. 1 to 16.

58. A compound according to claim **57** having an amino acid sequence selected from SEQ. ID. NOS. 1 to 6.

59. A compound according to claim **1** wherein Xaa_3 is Ala or Gln, Xaa_4 is Ala or Asn and Xaa_5 is Ala or Val.

60. A compound according to claim **59** wherein Xaa_4 is Ala.

61. A compound according to claim **60** wherein Xaa_3 is Ala.

62. A compound according to claim **61** wherein Xaa_5 is Ala.

63. A compound according to claim **59** wherein Xaa_3 is Gln.

64. A compound according to claim **63** wherein Xaa_5 is Val.

65. A compound according to claim **64** wherein Xaa_4 is Asn.

66. A compound according to claim **1** wherein X_4 is ArgLeuAla.

67. A compound according to claim **1** wherein X_5 is PheLeu.

68. A compound according to claim **1** wherein X_6 is ArgSerSerGlyTyr (SEQ. ID. NO. 48), HisSerSerGlyTyr (SEQ. ID. NO. 50), ArgSer or HisSer.

69. A compound according to claim **1** wherein X_2 is AsnThrAlaThr (SEQ. ID. NO. 59), AsnValAlaThr (SEQ. ID. NO. 60), AsnLeuAlaThr (SEQ. ID. NO. 61) or AsnMetAlaThr (SEQ. ID. NO. 62).

70. A compound according to claim **69** wherein X_2 is AsnThrAlaThr (SEQ. ID. NO. 59) or AsnValAlaThr (SEQ. ID. NO. 60).

71. A compound according to claim **1** wherein Xaa_1 is Cys.

72. A compound according to claim **72** wherein Xaa_2 is Cys.

73. A compound according to claim **73** wherein Xaa_1 and Xaa_2 form a disulfide bridge.

74. A compound according to claim **1** wherein X_2 is absent.

75. A compound according to claim **74** wherein Xaa_1 is absent.

76. A compound according to claim **75** wherein Xaa_2 is Ala.

77. A pharmaceutical composition comprising a compound of any one of claims **1**, **56**, **57** or **58** and a pharmaceutically acceptable carrier.

78. A method of treating diabetes in a subject in need of treatment which comprises administering said subject a therapeutically effective amount of a compound of any one of claims **1**, **56**, **57**, or **58**.

79. A method according to claim **78** wherein said diabetes is Type I diabetes.

80. A method according to claim **78** wherein said diabetes is Type II diabetes.

81. A method of beneficially regulating gastrointestinal motility in a subject comprising administering to said subject a therapeutically effective amount of a compound of any one of claims **1**, **56**, **57**, or **58**.

82. A method according to claim **81** wherein said beneficial regulation of gastrointestinal motility comprises delaying gastric emptying.

* * * * *

US005950830A

United States Patent [19]

Trigger

[11] **Patent Number: 5,950,830**

[45] **Date of Patent: *Sep. 14, 1999**

[54] **PACKAGING FOR PATCHES**

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[52] **U.S. Cl.** **206/440**; 424/449

[58] **Field of Search** 206/438, 440, 206/461, 467, 471; 424/449; 53/478, 467, 471

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Assistant Examiner—Luan K. Bui
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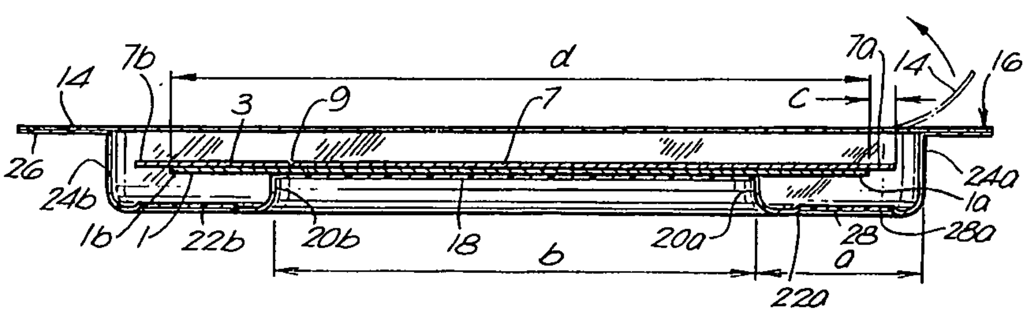
[57] **ABSTRACT**

A package (**12, 14**) contains a transdermal patch (**1**) releasably mounted on a release liner (**3**) larger than the patch so that a peripheral edge (**1***a*, **1***b*) of the patch is spaced from the edge of the release liner (**3**) by a border (**7**). The package (**12, 14**) is shaped and being sufficiently rigid that said edge of the patch is maintained spaced from the package. The package comprises a container (**12**) and a closure (**14**). The container has a recess containing the patch (**1**) and the release liner (**3**), and the recess has a base (**18, 22**) which includes a raised portion (**18**) located in the base so that, with the patch (**1**) facing the base, the edge (**1***a*, **1***b*) of the patch is maintained spaced from the base in any position of the release liner within the recess.

8 Claims, 3 Drawing Sheets



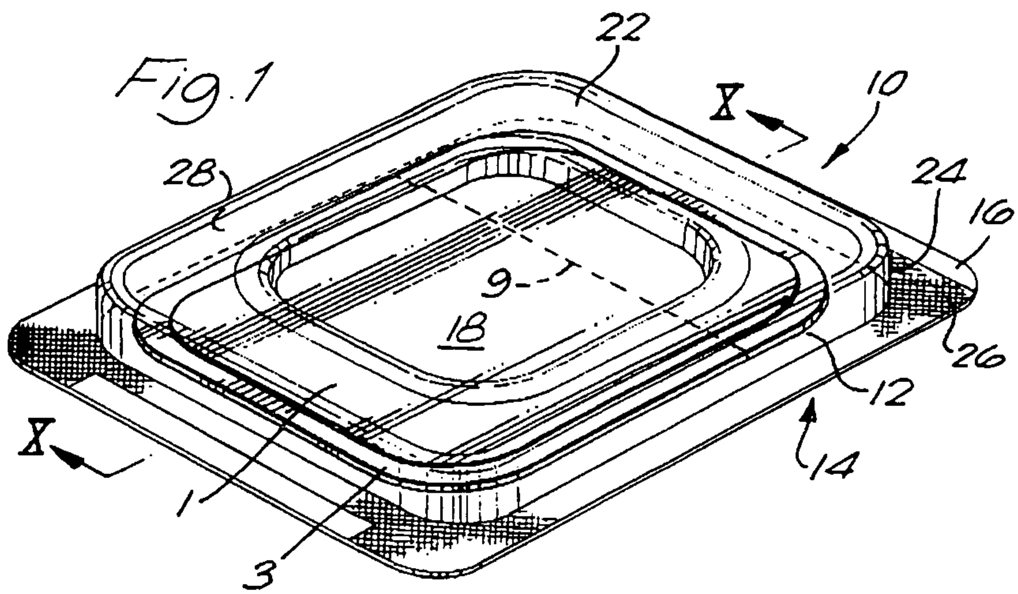


Fig.1

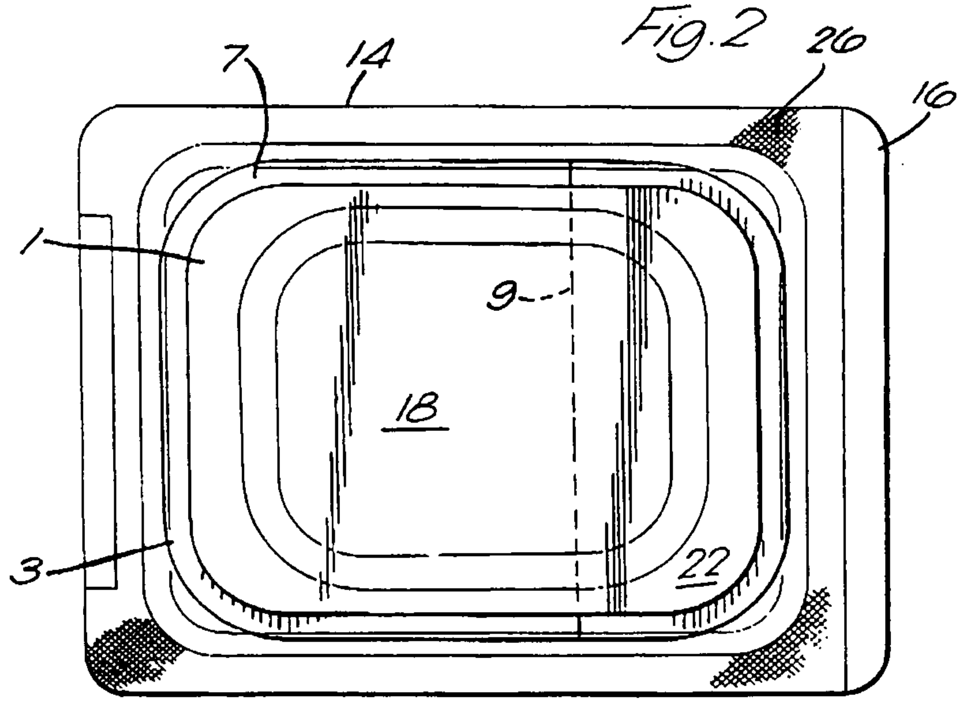
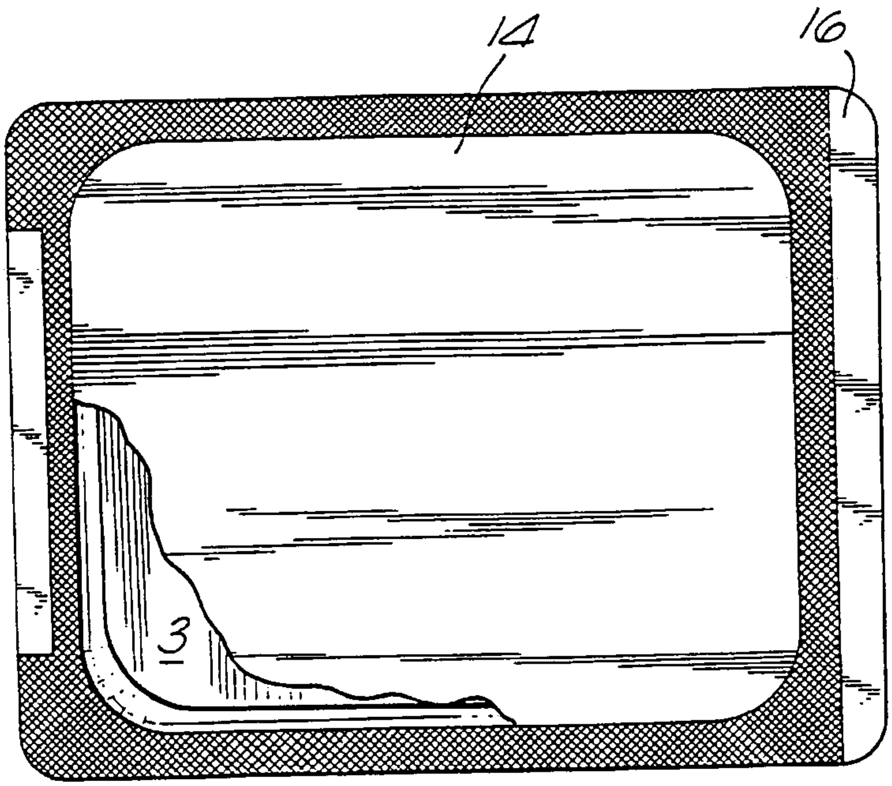


Fig.2

Fig. 3



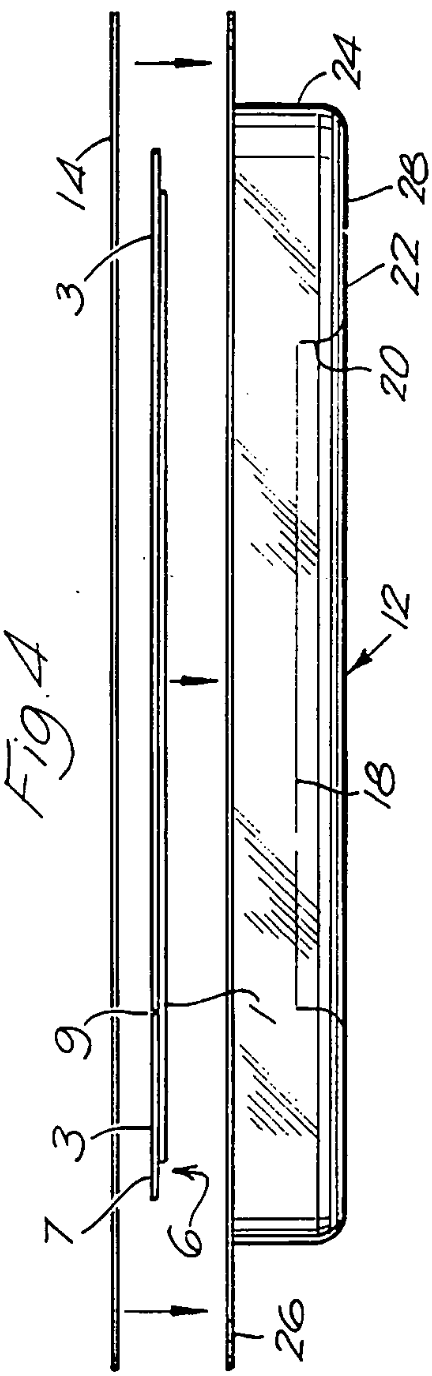

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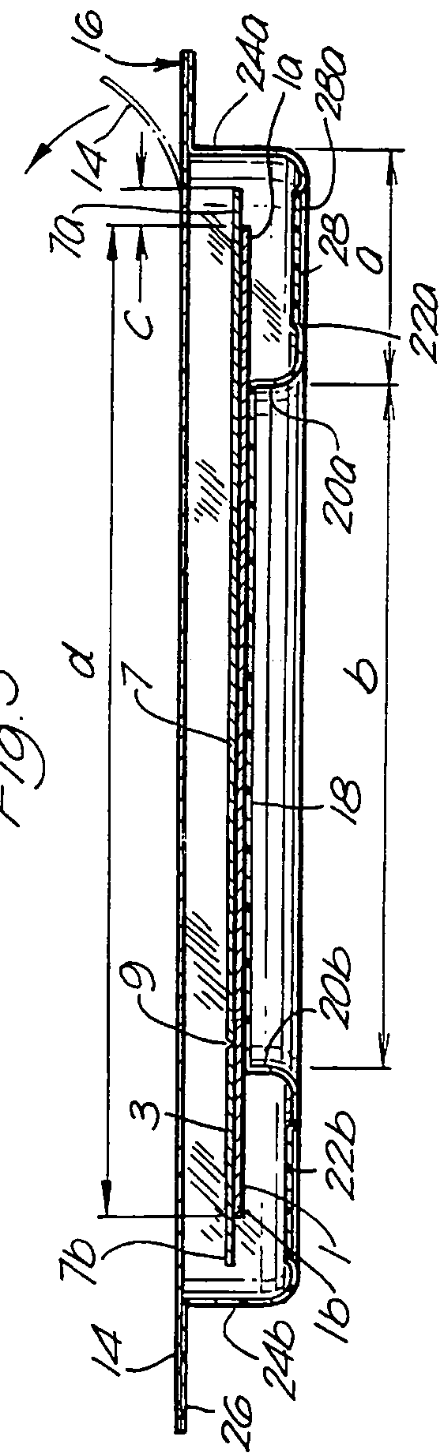
Fig. 4


16
24a
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14
7a
c
28
a
22a
20a
d
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18
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20b
3
22b
7b
1
1b
14
24b
26

Fig. 5

PACKAGING FOR PATCHES

TECHNICAL FIELD

The present invention relates to a package containing a patch for application to the skin, particularly but not exclusively a transdermal patch. The present invention also relates to a kit for making such a package and to the use of such a package.

BACKGROUND OF THE INVENTION

Medical transdermal patches have many uses, for example in hormone replacement, anti-anginal, travel sickness and smoking cessation therapy. Although various types of transdermal patches have been used, it is now generally preferred to use a patch which has a backing sheet and one or more layers, including an adhesive layer which attaches the patch to the skin. The adhesive layer is typically a viscous liquid containing at least one active component. The patch is supplied on a release-liner covering the adhesive, which is peeled off immediately before applying the patch to the skin. The active component then passes through the skin.

It is known to package such patches in a polymer or multi-layer laminate envelope or sachet. WO 95/00122 (published Jan. 5, 1995, after the priority date now claimed), shows an example of this, in which a release liner carrying four patches is enclosed in a resealable bag made of plastic film or foil laminate. A modified package is known from Japanese Utility Model publication 1-155428 (accepted publication no. 4-51782) in which the patch is mounted on a release liner provided with raised dimples to minimise contact between the envelope or sachet and the patch. A similar package has been marketed, for example, by Ortho-Cilag under the name Evorel™. It has been found that although the raised dimples provide a limited degree of protection, they do not prevent pressure on the patch due to handling, leading to oozing of the viscous adhesive which forms a sticky ring around the patch. This results in a tendency for the patch to stick to the envelope or sachet.

It is also known to provide a blister-pack in which a patch attached to a release liner, which is the same size as the patch itself, is located in a shallow blister formed between a plastic moulding and a foil lidding. A rib is formed around the blister, to increase rigidity of the otherwise fragile pack which is susceptible to curling. Such a patch is believed to have been marketed under the name TRIALSAT™ by Beta Pharmaceuticals.

Use of a release liner which is the same size as the patch means that the only sticky part of the patch is at the periphery, though adhesive can ooze around the edges of the patch. However, because the patch and release liner are the same size, it can be difficult and messy to separate the two. It may be undesirable that the active component is touched by a user, except at the intended application point. Furthermore, the need for a reinforcing rib around the blister increases the overall size of the package, so that the package occupies a much larger volume than the patch itself.

SUMMARY OF THE INVENTION

The present invention seeks to overcome the above problems, by providing a novel package for a patch in which the patch is substantially prevented from sticking to the package. In addition the release liner can be more readily separable from the patch and the package may be rigid enough to provide a degree of mechanical protection for the patch without the overall size of the package necessarily becoming excessive in comparison to the size of the patch.

Accordingly, the present invention provides a package containing a patch for application to the skin having an adhesive layer by which the patch is releasably mounted on a release liner larger than the patch, the peripheral edge of the patch being spaced from the peripheral edge of the release liner by a border region of the release liner, the package being sufficiently rigid and being shaped so that said peripheral edge of the patch is substantially prevented from contacting any part of the package. This prevents any adhesive which may emerge at the edge of the patch, during normal storage and transport of the package, from sticking the patch to the package.

Desirably, the patch and release liner can move freely as a unit both in the lateral direction (i.e. in the plane of the patch) and in the vertical direction (i.e. perpendicular to the plane of the patch) within the package.

The border region of the release liner preferably extends continuously around the patch, but the edge of the patch may coincide with the edge of the release liner at one or more zones, provided that the condition is met that the edge of the patch cannot contact the package.

The present invention also provides a kit of parts for use in making such a package.

Preferably the package comprises a container closed by a closure, the container having a central portion at a first depth from the closure joined to an outer portion at a second depth from the closure deeper than the first depth, the outer portion being provided with an outer wall to which the closure is attached, the dimensions being such that with the patch supported on the central portion with the release liner uppermost towards the closure, the edge of the patch lies above said deeper outer portion.

In one preferred form, the patch is mounted on a release liner larger in area than the patch so that the periphery of the patch is spaced inwardly from the periphery of the release liner and the package comprises a container in the form of a moulded sheet and a closure sheet, the container having a flange region surrounding a recess containing the patch and release liner, the recess having a base which includes at least one raised portion located in the base so that, with the patch facing towards the base, the periphery of the patch is maintained spaced from the base in any position of the release liner within the recess, the closure sheet being secured to the flange region.

Preferably the raised portion or portions are spaced from the periphery of the recess, and may take the form of a single raised central region smaller than the patch, or a plurality of raised regions, such as ribs, dispersed over a central area smaller than the patch.

The invention is particularly suitable for a package containing a single patch, but may also for example be used for a package containing a plurality of patches mounted on a single release liner.

DESCRIPTION OF SPECIFIC EMBODIMENT

An embodiment of the invention will now be described by way of example with reference to the following drawings in which:

FIG. 1 is a perspective view from underneath of a package containing a patch which is an embodiment of the invention;

FIG. 2 is an underneath plan view of the package of FIG. 1;

FIG. 3 is a partially cut-away top plan view of the package of FIG. 1;

FIG. 4 is an exploded side view of the package of FIG. 1; and

FIG. 5 is a section along X—X in FIG. 1.

Referring to FIGS. 1–3, a self-adhesive transdermal patch 1 consists of a backing and a thin adhesive layer (not separately shown) of viscous liquid containing an active component, such as a pharmaceutical, to be transported across the skin. This patch 1 is releasably mounted on a release liner 3 by the adhesive layer, with the adhesive layer interposed between the backing and the liner 3. The release liner 3, which may be e.g. a metallized plastics sheet, is larger than the patch 1 which is mounted on a central patch-carrying portion of one surface 6 of the release liner 3 surrounded all around by a border 7. The whole length of the peripheral edge of the patch 1 is inwardly spaced from the edge of the release liner 3. The border 7 is sufficiently wide to facilitate removal of the patch 1 from the release liner 3 by the user and to retain any adhesive escaping from under the patch on the surface 6 substantially to prevent adhesive reaching the edge of the release liner 3.

The release liner 3 is divided into two parts about two-thirds along its length by a slit 9 across its entire width, the two parts being held together by the patch 1. To remove the patch 1 from the release liner 3, the patch 1 is folded back on itself at the slit 9 and a first (e.g. smaller) part of the release liner 3 may be removed. The thus exposed drug-in-adhesive layer of the patch 1 may be applied to the intended site of application on the skin and the second part of the release liner 3 removed, application of the patch 1 being completed by applying pressure to the backing of the patch 1. Thus, in this way, the patch 1 may be applied without any handling of the adhesive surface.

In this specific embodiment, the patch 1 is substantially rectangular, with rounded corners, about 5 cm wide by 6 cm long and the border 7 is uniformly approximately 4 mm wide all around the patch 1, with the slit in the release liner 3 about 22 mm from a short side. Of course a variety of shapes and sizes of patch 1 and release liner 3 may be used depending on the particular application.

The patch 1 mounted on the release liner 3 is stored in a package 10 comprising a container 12 to which a closure 14 is adhesively sealed. In this embodiment the interior of the package 10 is hermetically sealed by the seal of the closure 14, and may be filled with an inert gas, such as nitrogen. The gas pressure helps to prevent the package 10 being compressed. The package 10 is sufficiently rigid to retain its shape and resist deformation under pressures and loads normally encountered in transport and storage. In this embodiment the container is made of transparent moulded plastics material sheet, and the closure 14 is flat, made from flexible metal (aluminium) foil, the package being constructed in the manner of a so-called blister pack with a recess containing the patch and release liner.

As can best be seen from FIGS. 4 and 5, the closure 14 and container 12 can be separated simply by peeling them apart, starting at a tab portion 16 where they are not sealed together. Other typical examples of alternative flexible materials which may provide a peelable closure are plastics sheet, or a laminate comprising aluminium and/or plastics material and/or paper, the closure in this case being flexible and sealed by a suitable adhesive or heat-sealable layer. However, as will be understood, a variety of materials may be used depending on the degree of rigidity or opacity required; for example the container 12 may be made of metal. Opacity may be desirable, to exclude light.

The container 12 has a substantially flat horizontal central portion 18 typically occupying a major part of the area of the container 12. This is joined by a first wall 20 to a surrounding outer portion 22 of greater depth from the closure 14. The outer portion 22 is closed by an outer wall 24, the top of which joins a flange 26 to which the closure 14 is sealed. The tab portion 16 described above is at a part of the flange 26. In this embodiment, the top regions of the first, inner, wall 20, and the outer wall 24 are substantially vertical to provide clearly defined boundaries to the outer portion 22, whereas the bottoms of the first, inner, wall 20 and outer wall 24 curve smoothly to form the bottom 28 of the outer portion 22. With this construction, the outer portion 22 together with the inner and outer walls 20,24 serve to increase the overall rigidity of the package 10. Increased rigidity is provided by a shallow upstand 28*a* in the outer portion 22.

When the package is in its normal rest position with the closure sheet 14 upwards, the centre of the patch 1 rests on the platform provided by the central portion 18, with the release liner 3 uppermost towards the closure 14. The border 7, described above, prevents the edge of the patch 1 itself contacting the outer wall 24. The central portion 18 is smaller than the patch 1 so that the whole length of the edge of the patch must lie above the trough formed by the outer portion 22.

The depth of the central portion 18 from the closure 14 is greater than the combined thickness of the patch 1 and release liner 3 by an amount sufficient to allow the patch and liner to move freely between contact with the closure 14 and contact with the central portion 18, thus preventing the patch 1 being pinched or compressed between the closure 14 and the central portion 18 in normal conditions, thereby reducing the tendency for the adhesive to be squeezed out from under the patch 1. In particular, the package should withstand the weight of several (e.g. at least a dozen) such packages to enable stacking for transport. Typical pressures which the container should withstand without the patch being compressed are 100 grams spread over 1 square centimeter, and 200 grams spread over the entire patch.

The depth of the outer portion 22 from the closure 14 is greater than that of the central portion 18 by an amount sufficient to prevent the edges 1*a*,1*b* of the patch 1, or adhesive escaping outwardly therefrom, contacting the bottom 28.

In this embodiment, the central portion 18 is about 2.5 mm deep from the closure 14 and the outer portion 22 is about 6.5 mm deep from the closure 14 so the difference is about 4 mm. If the release liner 3 and package 10 are sufficiently rigid, the difference in depths and/or the overall depth may be reduced without the edges 1*a*,1*b* of the patch contacting the bottom 28.

As can be seen from the section shown in FIG. 5, the sum of the width a of the outer portion 22*a* at a first side and the width b of the central portion 18 is less than the sum of the width c of the border 7*a* of the release liner at the first side and the width d of the patch 1. Thus the edge 1*b* of the patch 1, where adhesive may escape, is made to remain within the second outer portion 22*b* of the container, away from the inner wall portion 20*b*, by the edge of the first border portion 7*a* abutting the first outer wall portion 24*a* or by the edge of the opposite border portion 7*b* contacting the wall portion 24*b*. This is true for any cross-section, and for all locations and orientations to which the patch 1 can move within the package 10, so that no part of the peripheral edge of the patch 1 can contact the inner wall 20.

In this embodiment, with a patch of the dimensions given above, the container 12 has a central portion 18 38 mm wide, bordered by the outer portion 22 which is 13 mm wide to

give a total overall width of 64 mm. The central portions 44 mm long, bordered by outer portion **22** which is 15 mm long to give an overall length of 74 mm. The bottom **28** of the outer portion **22** curves smoothly into the inner wall **20** and outer wall **24** via arcuate sections respectively having radii of curvature of approximately 4 mm. The same package **10** can accommodate alternative sizes and shapes of patches if desired, provided the edges of the patch are prevented from contacting the walls of the package as described above.

As described above, the inner and outer walls **20**,**24** in this embodiment are smooth and continuous, and the boundaries of the outer portion **22** are clearly defined. However, as will be appreciated, the outer wall **24** (for example) may be interrupted by ribs or the like which obstruct movement of the release liner, the central portion **18** need not be flat, and the transition between the central portion **18** and the outer portion **22** need not be so abrupt. The central portion **18** may be a continuous upstanding rib or a plurality of upstanding areas. The important requirement in these embodiments is that the central portion supports the patch, preferably over a major part of the area of the patch, so that the edges of the patch cannot contact the central portion, or other parts of the container. The width a of the outer portion is then measured from a point (e.g. a rib) **24***a* (corresponding to the outer wall **24**) which obstructs movement of the release liner **3** to the first point **20***a* where the depth is such that the central portion can contact the patch **1**, and the effective width b of the central portion is measured therefrom to the furthest point **20***b* at which the depth is such that the central portion **18** can contact the patch **1**. In all cases, the effective width b of the central portion **18** along any given section is less than the corresponding width d of the patch **1**.

Furthermore, if, as in the above described embodiment, the release liner **3** is not circular, the dimensions of the package **10** are usually chosen so that the release liner **3** cannot rotate within the package **10**; the central portion **18** should be unable to contact the edges **1***a*,**1***b* of the patch **1** in any location or orientation of the patch **1** possible within the package **10**.

The invention is applicable to the packaging of medical, veterinary and non-medical and non-veterinary patches for application to human or animal skin.

I claim:

1. A package containing a patch for application to the skin, the patch including a backing sheet, an adhesive layer, on said backing sheet, having a first surface and a second surface, and a release liner sized larger than said backing sheet and attached to said second surface of said adhesive layer, the packaging comprising:

a container, of molded material, having a predetermined shape defining a recess sized to contain said patch, said recess being defined by a base wall and a peripheral wall upstanding from said base wall;

a flexible closure sealed to said container so as to cover over said recess;

said base wall of said recess having a first, outer, peripheral region adjacent said peripheral wall, and having a second region surrounded, by said outer peripheral region and being at least partly raised relative to said outer peripheral region so as to provide for said patch a support surface closer to said closure than to said outer peripheral region;

said patch being disposed in said package with said backing sheet lying against said support surface;

each of said recess, said second region of said base wall, said release liner, and said backing sheet sized such that in all positions of said patch within said package, a peripheral edge of said backing sheet lies above and spaced from said outer peripheral region of said base wall and does not contact said support surface;

said closure and said support surface being spaced apart by a distance substantially greater than a total thickness of said patch whereby said patch is prevented from being compressed between said closure and said support surface when said container retains its said predetermined shape and adhesive from said patch is prevented from contact with said package.

2. A package according to claim **1** wherein:

said container is filled with inert gas.

3. A package according to claim **1**, wherein said container is hermetically sealed to said closure.

4. A package according to claim **1**, wherein said container is fabricated from a plastic material.

5. A package according to claim **1**, wherein said closure is fabricated from a metal foil material.

6. A package according to claim **1**, wherein said adhesive layer includes a pharmaceutical agent.

7. A package according to claim **1**, wherein said adhesive layer includes a beneficial agent.

8. A package according to claim **1**, wherein said patch is a medicated transdermal patch.

* * * * *

US005776477A

United States Patent [19]

Ryder

[11] Patent Number: 5,776,477

[45] Date of Patent: Jul. 7, 1998

[54] ORGANIC INSECT REPELLENT

[76] Inventor: Kathleen A. Ryder, 208 Chestnut St., Middleburg, Va. 22117

[21] Appl. No.: 676,419

[22] Filed: Jul. 8, 1996

[51] Int. Cl.^{6} A01N 25/04

[52] U.S. Cl. 424/405; 424/406; 424/195.1; 424/DIG. 10; 514/919

[58] Field of Search .... 424/405, 406, 424/195.1, DIG. 10; 514/783, 919

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Primary Examiner—Neil S. Levy
Attorney, Agent, or Firm—Longacre & White

[57] ABSTRACT

An environmentally safe, topical pest repellent is described. The repellent comprises a variable mixture of natural ingredients including pennyroyal herb, tansy herb, calendula, citronella, pyrethrin and aloe vera. A method is provided to produce tinctures which are combined to form a composition for application to skin of animals such as horses to repel pests such as flies, mosquitos, ticks, and other insects. The composition may be applied to animals by spraying or other suitable devices and carriers.

2 Claims, No Drawings

ORGANIC INSECT REPELLENT

BACKGROUND OF THE INVENTION

a) Field of the Invention

This invention relates to a composition for the treatment of animals, and more particularly relates to a new formulation of natural ingredients for repelling pests such as flies and mosquitos from household and domestic animals.

b) Description of Related Art

Insect infestation of animals and humans has been a problem through the ages. While improvements in hygiene and sanitation have eliminated the problem for human beings in most parts of the world, household pets and domestic animals still are subject to insect infestation. While the problem is especially apparent to owners of household pets such as cats and dogs since these animals often have a great deal of contact with their owners, domestic animals also suffer from the harmful effects of these pests due to their prolonged exposure.

Both household and domestic animal owners dedicate substantial time and money each year in an effort to protect animals against pests such as insects. One of the major expenditures is in the area of fly repellents for domestic animals. A variety of products are commercially available for repelling flies including shampoos, sprays, powders and the like.

An important concern of pet owners is the hazardous character of the chemicals used in the fly repellents. Not only is the owner concerned about the effect such chemicals will have on the health of his pet, but also the effect of such chemicals on the health of the members of the community who are in contact with the animal. In view of this hazard, animal owners and handlers are in need of a new treatment for their animals that eliminates flies and other insects, and yet is not hazardous to the animal or persons coming into contact with the animal.

U.S. Pat. No. 5,102,622, which is incorporated herein by reference, provides a discussion of the various efforts to overcome the harmful effects of chemicals and to develop suitable organic repellents. However, the solutions discussed in this patent lack the necessary skin conditioning and insect repelling characteristics necessary for a suitable and efficient composition.

SUMMARY OF THE INVENTION

The present invention provides a novel composition for the treatment of animals and particularly domestic animals such as horses. The pest-repellent composition of the invention is formulated of natural ingredients which is easily formulated with commercially available materials using conventional mixing techniques in a novel manner. Also, the pest-repellent composition of the invention is relatively inexpensive.

The invention relates to a repellent composition comprising various mixtures of pennyroyal herb, tansy herb, vinegar, isopropyl alcohol, calendula, pure Ceylon citronella oil, pyrethrin and pure aloe vera juice.

The composition is produced by making two separate tinctures and mixing them together with small amounts of flower oil and an insecticide. The first tincture is made by drying 3 parts pennyroyal and 1 part tansy herb. These ingredients are mixed with isopropyl alcohol and acetic acid and agitated for a predetermined period of time, then the ingredients are strained. The second tincture, which soothes and promoted healing of skin, is made by mixing calendula (flowers) in a container having acetic acid and alcohol. The container is sealed and agitated. The calendula is strained by filter the liquid into a separate container. These first and second tinctures are then mixed together and combined with an insect repellent and an active insecticidal constituent of a flowering plant. This mixture is then stirred constantly while being poured into separate containers for use.

In the preferred embodiment, the composition of this invention is applied to animals by a spray device, but other suitable forms of administering the solution, e.g. soap, shampoo, lotion, etc., may be used.

The foregoing combination of organic ingredients and the method of producing the composition of this invention provides a non-irritating insect repellent that has skin conditioning properties and a pleasant fragrance. Other benefits and advantages of the novel repellent composition of the invention will be apparent from the following description.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As described herein, the invention is a composition suitable for use as a topical solution for living beings and having pest repellent properties. This invention is not intended to be limited to insects but has been shown to repel a variety of creatures which prosper at the expense of a host animal.

The composition of this invention is produced by making two separate tinctures and mixing them together with small amounts of flower oil and an insecticide. The first tincture is made by drying about 3 parts pennyroyal to about 1 part tansy herb. These herbs are placed in a container and combined with about 2 parts isopropyl alcohol to about 3 parts acetic acid. The container is sealed and agitated periodically for a number of days. After the predetermined period of time, the herbs are stained by filtering the liquid into a separate container.

The second tincture, which soothes and promotes healing of the skin, is made by placing calendula (flowers) in a container having two parts acetic acid to one part alcohol. The container of the second tincture is sealed and agitated periodically for a number of days. After the predetermined period of time, the calendula is strained by filtering the liquid into a separate container.

The third step involves mixing the first and second tinctures together while adding an insect repellent (pure Ceylon citronella oil) and an active insecticidal constituent of a flowering plant (pyrethrin) to the mixture. Also added is pure aloe vera juice; a constituent that is not only soothing and healing to the skin, but reduces the astringency of the isopropyl alcohol. This mixture is then stirred constantly while being poured into containers.

The foregoing mixture is applied to the external surfaces of animals to repel flies, mosquitos, etc. while maintaining a pleasant odor and acceptable toxicity due to its organic nature.

In a particularly preferred composition and method, the constituents of the composition are as follows. These ingredients and relative amounts are set forth by way of example only and are not intended to limit the invention in any manner.

Tincture 1

1½ pounds of pennyroyal (hedeoma pulegioides)
½ pound of tansy herb (tanacetum)
3 gallons acetic acid (vinegar)
2 gallons 70% isopropyl alcohol

Tincture 2

1 pound calendula

2 gallons acetic acid (vinegar)

1 gallon 70% isopropyl alcohol

The first tincture is made by drying the pennyroyal and tansy herbs and placing them in a container. The isopropyl alcohol and acetic acid are added to the herbs. The container is then sealed and the mixture is agitated daily for three weeks. After the third week, the herbs are stained by filtering the liquid into a separate container.

The second tincture is made by placing the calendula (flowers) in a container having the acetic acid and alcohol. The container is sealed and agitated for three weeks. After the third week, the calendula is strained by filter the liquid into a separate container.

Next the first and second tinctures are mixed together adding about 6 ounces of pure Ceylon citronella oil and about 5 ounces of pyrethrin. An amount (about 8 ounces in this example) of pure aloe vera may also be added at this point. This mixture is then stirred constantly while being poured into containers.

The above description shows that the present invention provides a novel formulation for the treatment of animals and especially the treatment of domestic animals against flies. Of course, the exact amount or ratio of each ingredient may be varied within a reasonable range without departing from the spirit of this invention. The composition of the invention is easily formulated from natural ingredients which are commercially available.

Conventional mixing techniques may be employed in the formulation of the composition. It will be apparent that various modifications can be made in the particular formulation described in detail above within the scope of the invention. For example, other ingredients may be incorporated in the formulation provided they do not have a del-

eterious effect on the performance characteristics of the composition of the invention. It may be desirable in some applications to change the odor, color, viscosity or other aspects of the composition. In addition, the inert vehicle may be different to meet specific requirements, e.g. a powder. Therefore, the scope of the invention is to be limited only by the following claims.

I claim:

1. An organic composition for the treatment of animals against pestilent beings, said composition comprising:

a first amount of pennyroyal, tansy, acetic acid and isopropyl alcohol, said pennyroyal and tansy is derived by mixing pennyroyal herb and tansy herb with said acetic acid and said isopropyl alcohol in a first tincture, a ratio of pennyroyal herb to tansy herb is between 4:1 and 2:1 and said isopropyl alcohol is between 40% and 80% by weight of said acetic acid, wherein said isopropyl alcohol serves to enhance beneficial properties of said pennyroyal herb and said tansy herb;

a second amount of calendula, acetic acid and isopropyl alcohol, said calendula derived by mixing calendula herbs with isopropyl alcohol in a second tincture, said isopropyl alcohol is between 40% and 80% by weight of said acetic acid, said isopropyl alcohol serving to enhance beneficial properties of said calendula, wherein a ratio of calendula herb to tansy herb is about 2:1;

a third amount of citronella oil which is between about 2% and 7% by weight of said composition, and

a fourth amount of pyrethrin which is between about 1% and 6% by weight of said composition.

2. A composition according to claim 1, wherein said first amount is between 50% and 75% by weight of said composition.

* * * * *

US006585764B2

(12) **United States Patent**
Wright et al.

(10) **Patent No.: US 6,585,764 B2**
(45) **Date of Patent: Jul. 1, 2003**

(54) **STENT WITH THERAPEUTICALLY ACTIVE DOSAGE OF RAPAMYCIN COATED THEREON**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/874,117**

(22) Filed: **Jun. 4, 2001**

(65) **Prior Publication Data**

US 2001/0027340 A1 Oct. 4, 2001

Related U.S. Application Data

(63) Continuation of application No. 09/061,568, filed on Apr. 16, 1998, now Pat. No. 6,273,913.

(60) Provisional application No. 60/044,692, filed on Apr. 18, 1997.

(51) **Int. Cl.**[7] **A61F 2/06**

(52) **U.S. Cl.** **623/1.42**

(58) **Field of Search** 623/1.15, 1.39, 623/1.42, 1.4; 427/2.1–2.31

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(57) **ABSTRACT**

Delivery of rapamycin locally, particularly from intravascular stent, directly from micropores in the stent body or mixed or bound to a polymer coating applied on stent, to inhibit neointimal tissue proliferation and thereby prevent restenosis. This invention also facilitates the performance of the stent in inhibiting restenosis.

20 Claims, 2 Drawing Sheets

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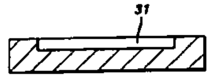

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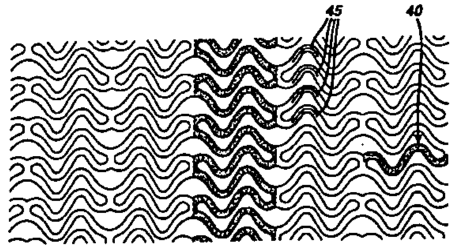

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FIG. 1

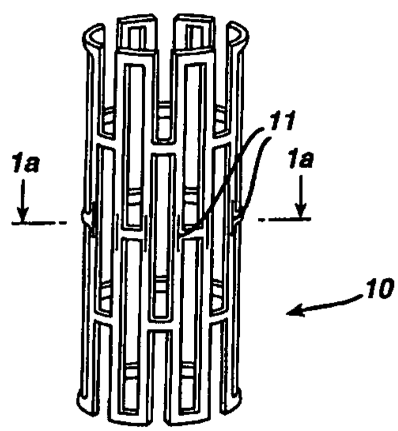


FIG. 1a

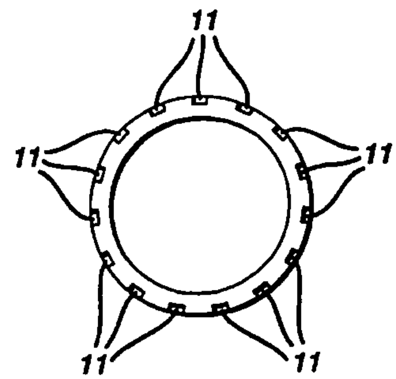


FIG. 2a

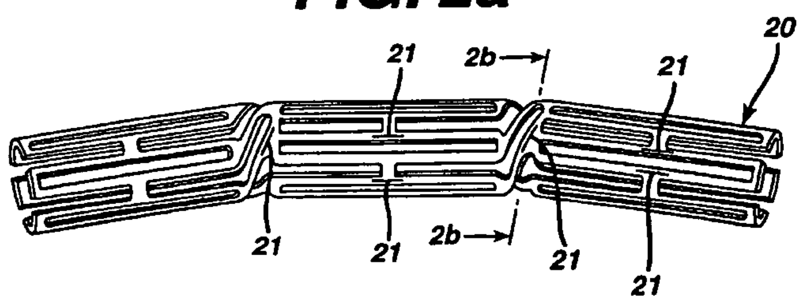


FIG. 2b

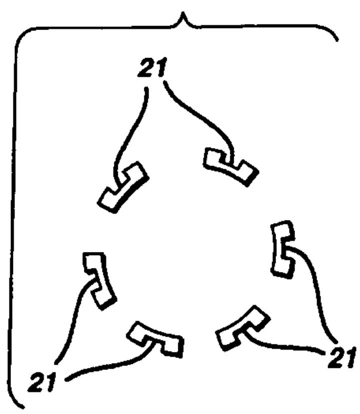


FIG. 3a

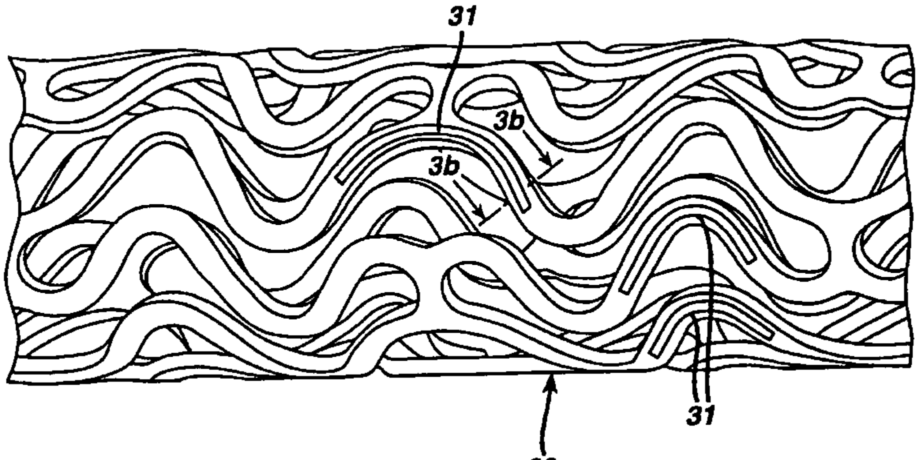


FIG. 3b

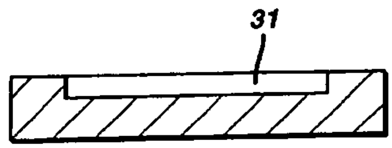
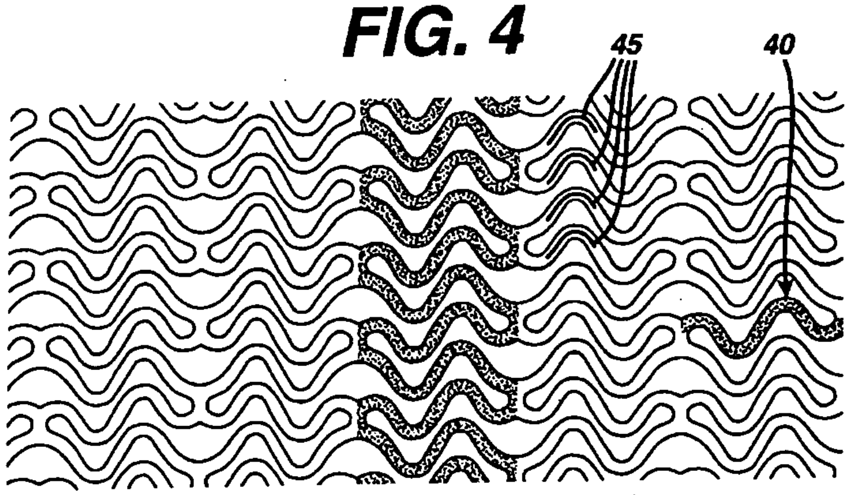


FIG. 4



STENT WITH THERAPEUTICALLY ACTIVE DOSAGE OF RAPAMYCIN COATED THEREON

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation application of U.S. application Ser. No. 09/061,568, filed Apr. 16, 1998, now issued as U.S. Pat. No. 6,273,913, which claims the benefit of U.S. Provisional Application No. 60/044,692, filed Apr. 18, 1997.

FIELD OF THE INVENTION

Delivery of rapamycin locally, particularly from an intravascular stent, directly from micropores in the stent body or mixed or bound to a polymer coating applied on stent, to inhibit neointimal tissue proliferation and thereby prevent restenosis. This invention also facilitates the performance of the stent in inhibiting restenosis.

BACKGROUND OF THE INVENTION

Re-narrowing (restenosis) of an artherosclerotic coronary artery after percutaneous transluminal coronary angioplasty (PTCA) occurs in 10–50% of patients undergoing this procedure and subsequently requires either further angioplasty or coronary artery bypass graft. While the exact hormonal and cellular processes promoting restenosis are still being determined, our present understanding is that the process of PTCA, besides opening the artherosclerotically obstructed artery, also injures resident coronary arterial smooth muscle cells (SMC). In response to this injury, adhering platelets, infiltrating macrophages, leukocytes, or the smooth muscle cells (SMC) themselves release cell derived growth factors with subsequent proliferation and migration of medial SMC through the internal elastic lamina to the area of the vessel intima. Further proliferation and hyperplasia of intimal SMC and, most significantly, production of large amounts of extracellular matrix over a period of 3–6 months results in the filling in and narrowing of the vascular space sufficient to significantly obstruct coronary blood flow.

Several recent experimental approaches to preventing SMC proliferation have shown promise althrough the mechanisms for most agents employed are still unclear. Heparin is the best known and characterized agent causing inhibition of SMC proliferation both in vitro and in animal models of balloon angioplasty-mediated injury. The mechanism of SMC inhibition with heparin is still not known but may be due to any or all of the following: 1) reduced expression of the growth regulatory protooncogenes c-fos and c-myc, 2) reduced cellular production of tissue plasminogen activator; are 3) binding and dequestration of growth regulatory factors such as fibrovalent growth factor (FGF).

Other agents which have demonstrated the ability to reduce myointimal thickening in animal models of balloon vascular injury are angiopeptin (a somatostatin analog), calcium channel blockers, angiotensin converting enzyme inhibitors (captopril, cilazapril), cyclosporir A, trapidil (an antianginal, antiplatelet agent), terbinafine (antifungal), colchicine and taxol (antitubulin antiproliferatives), and c-myc and c-myb antinsense oligonucleotides.

Additionally, a goat antibody to the SMC mitogen platelet derived growth factor (PDGF) has been shown to be effective in reducing myointimal thickening in a rat model of balloon angioplasty injury, thereby implicating PDGF directly in the etiology of restenosis. Thus, while no therapy has as yet proven successful clinically in preventing restenosis after angioplasty, the in vivo experimental success of several agents known to inhibit SMC growth suggests that these agents as a class have the capacity to prevent clinical restenosis and deserve careful evaluation in humans.

Coronary heart disease is the major cause of death in men over the age of 40 and in women over the age of fifty in the western world. Most coronary artery-related deaths are due to atherosclerosis. Atherosclerotic lesions which limit or obstruct coronary blood flow are the major cause of ischemic heart disease related mortality and result in 500,000–600,000 deaths in the United States annually. To arrest the disease process and prevent the more advanced disease states in which the cardiac muscle itself is compromised, direct intervention has been employed via percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass graft (CABG).

PTCA is a procedure in which a small balloon-tipped catheter is passed down a narrowed coronary artery and then expanded to re-open the artery. It is currently performed in approximately 250,000–300,000 patients each year. The major advantage of this therapy is that patients in which the procedure is successful need not undergo the more invasive surgical procedure of coronary artery bypass graft. A major difficulty with PTCA is the problem of post-angioplasty closure of the vessel, both immediately after PTCA (acute reocclusion) and in the long term (restenosis).

The mechanism of acute reocclusion appears to involve several factors and may result from vascular recoil with resultant closure of the artery and/or deposition of blood platelets along the damaged length of the newly opened blood vessel followed by formation of a fibrin/red blood cell thrombus. Recently, intravascular stents have been examined as a means of preventing acute reclosure after PTCA.

Restenosis (chronic reclosure) after angioplasty is a more gradual process than acute reocclusion: 30% of patients with subtotal lesions and 50% of patients with chronic total lesions will go on to restenosis after angioplasty. While the exact mechanism for restenosis is still under active investigation, the general aspects of the restenosis process have been identified.

In the normal arterial will, smooth muscle cells (SMC) proliferate at a low rate (<0.1%/day; ref). SMC in vessel wall exists in a 'contractile' phenotype characterized by 80–90% of the cell cytoplasmic volume occupied with the contractile apparatus. Endoplasmic reticulum, golgi bodies, and free ribosomes are few and located in the perinuclear region. Extracellular matrix surrounds SMC and is rich in heparin-like glycosylaminoglycans which are believed to be responsible for maintaining SMC in the contractile phenotypic state.

Upon pressure expansion of an intracoronary balloon catheter during angioplasty, smooth muscle cells within the arterial wall become injured. Cell derived growth factors such as platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), etc. released from platelets (i.e., PDGF) adhering to the damaged arterial luminal surface, invading macrophages and/or leukocytes, or directly from SMC (i.e., BFGF) provoke a proliferation and migratory response in medial SMC. These cells undergo a phenotypic change from the contractile phenotyope to a 'synthetic' phenotype characterized by only few contractile filament bundles but extensive rough endoplasmic reticulum, golgi and free ribosomes. Proliferation/migration usually begins within 1–2 days post-

injury and peaks at 2 days in the media, rapidly declining thereafter (Campbell et al., In: *Vascular Smooth Muscle Cells in Culture,* Campbell, J. H. and Campbell, G. R., Eds, CRC Press, Boca Ration, 1987, pp. 39–55); Clowes, A. W. and Schwartz, S. M., Circ. Res. 56:139–145, 1985).

Finally, daughter synthetic cells migrate to the intimal layer of arterial smooth muscle and continue to proliferate. Proliferation and migration continues until the damaged luminal endothelial layer regenerates at which time proliferation ceases within the intima, usually within 7–14 days postinjury. The remaining increase in intimal thickening which occurs over the next 3–6 months is due to an increase in extracellular matrix rather than cell number. Thus, SMC migration and proliferation is an acute response to vessel injury while intimal hyperplasia is a more chronic response. (Liu et al., Circulation, 79:1374–1387, 1989).

Patients with symptomatic reocclusion require either repeat PTCA or CABG. Because 30–50% of patients undergoing PTCA will experience restenosis, restenosis has clearly limited the success of PTCA as a therapeutic approach to coronary artery disease. Because SMC proliferation and migration are intimately involved with the pathophysiological response to arterial injury, prevention of SMC proliferation and migration represents a target for pharmacological intervention in the prevention of restenosis.

SUMMARY OF THE INVENTION

Novel Features and Applications to Stent Technology

Currently, attempts to improve the clinical performance of stents have involved some variation of either applying a coating to the metal, attaching a covering or membrane, or embedding material on the surface via ion bombardment. A stent designed to include reservoirs is a new approach which offers several important advantages over existing technologies.

Local Drug Delivery from a Stent to Inhibit Restenosis

In this application, it is desired to deliver a therapeutic agent to the site of arterial injury. The conventional approach has been to incorporate the therapeutic agent into a polymer material which is then coated on the stent. The ideal coating material must be able to adhere strongly to the metal stent both before and after expansion, be capable of retaining the drug at a sufficient load level to obtain the required dose, be able to release the drug in a controlled way over a period of several weeks, and be as thin as possible so as to minimize the increase in profile. In addition, the coating material should not contribute to any adverse response by the body (i.e., should be non-thrombogenic, non-inflammatory, etc.). To date, the ideal coating material has not been developed for this application.

An alternative would be to design the stent to contain reservoirs which could be loaded with the drug. A coating or membrane of biocompatable material could be applied over the reservoirs which would control the diffusion of the drug from the reservoirs to the artery wall.

One advantage of this system is that the properties of the coating can be optimized for achieving superior biocompatibility and adhesion properties, without the addition requirement of being able to load and release the drug. The size, shape, position, and number of reservoirs can be used to control the amount of drug, and therefore the dose delivered.

DESCRIPTION OF THE DRAWINGS

The invention will be better understood in connection with the following figures in which

FIGS. 1 and 1*a* are top views and section views of a stent containing reservoirs as described in the present invention;

FIGS. 2*a* and 2*b* are similar views of an alternate embodiment of the stent with open ends;

FIGS. 3*a* and 3*b* are further alternate figures of a device containing a grooved reservoir; and

FIG. 4 is a layout view of a device containing a reservoir as in FIG. 3.

DETAILED DESCRIPTION OF THE INVENTION

Pharmacological attempts to prevent restenosis by pharmacologic means have thus far been unsuccessful and all involve systemic administration of the trial agents. Neither aspirin-dipyridamole, ticlopidine, acute heparin administration, chronic warfarin (6 months) nor methylprednisolone have been effective in preventing restenosis although platelet inhibitors have been effective in preventing acute reocclusion after angioplasty. The calcium antagonists have also been unsuccessful in preventing restenosis, although they are still under study. Other agents currently under study include thromboxane inhibitors, prostacyclin mimetics, platelet membrane receptor blockers, thrombin inhibitors and angiotensin converting enzyme inhibitors. These agents must be given systemically, however, and attainment of a therapeutically effective dose may not be possible; antiproliferative (or anti-restenosis) concentrations may exceed the known toxic concentrations of these agents so that levels sufficient to produce smooth muscle inhibition may not be reached (Lang et al., 42 Ann. Rev. Med., 127–132 (1991); Popma et al., 84 *Circulation,* 1426–1436 (1991)).

Additional clinical trials in which the effectiveness for preventing restenosis of dietary fish oil supplements, thromboxane receptor antagonists, cholesterol lowering agents, and serotonin antagonists has been examined have shown either conflicting or negative results so that no pharmacological agents are as yet clinically available to prevent post-angioplasty restenosis (Franklin, S. M. and Faxon, D. P., 4 Coronary Artery Disease, 232–242 (1993); Serruys, P. W. et al., 88 *Circulation,* (part 1) 1588–1601, (1993).

Conversely, stents have proven useful in preventing reducing the proliferation of restenosis. Stents, such as the stent **40**, seen in layout in FIG. **4**, balloon-expandable slotted metal tubes (usually but not limited to stainless steel), which when expanded within the lumen of an angioplastied coronary artery, provide structural support to the arterial wall. This support is helpful in maintaining an open path for blood flow. In two randomized clinical trials, stents were shown to increase angiographic success after PTCA, increase the stenosed blood vessel lumen and to reduce the lesion recurrence at 6 months (Serruys et al., 331 New Eng Jour. Med, 495, (1994); Fischman et al., 331 New Eng Jour. Med. 496–501 (1994). Additionally, in a preliminary trial, heparin coated stents appear to possess the same benefit of reduction in stenosis diameter at follow-up as was observed with non-heparin coated stents. Additionally, heparin coating appears to have the added benefit of producing a reduction in sub-acute thrombosis after stent implantation (Serruys et al., 93 Circulation, 412–422 (1996). Thus, 1) sustained mechanical expansion of a stenosed coronary artery has been shown to provide some measure of restenosis prevention, and 2) coating of stents with heparin has demonstrated both the feasibility and the clinical usefulness of delivering drugs to local, injured tissue off the surface of the stent.

Numerous agents are being actively studied as antiproliferative agents for use in restenosis and have shown some

activity in experimental animal models. These include: heparin and heparin fragments (Clowes and Karnovsky, 265 *Nature,* 25–626, (1977); Guyton, J. R. et. al. 46 Circ. Res., 625–634, (1980); Clowes, A. W. and Clowes, M. M., 52 Lab. Invest., 611–616, (1985) A. W. and Clowes, M. M., 58 Circ. Res., 839–845 (1986); Majesky et al., 61 Circ Res., 296–300, (1987); Snow et al., 137 Am. J. Pathol., 313–330 (1990); Okada, T. et al., 25 *Neurosurgery,* 92–898, (1989) colchicine (Currier, J. W. et al., 80 *Circulation,* 11–66, (1989), taxol (ref), agiotensin converting enzyme (ACE) inhibitors (Powell, J. S. et al., 245 Science, 186–188 (1989), angiopeptin (Lundergan, C. F. et al., 17 Am. J. Cardiol. (Suppl. B); 132B–136B (1991), Cyclosporin A (Jonasson, L. et. al., 85 Proc. Nati, Acad. Sci., 2303 (1988), goat-anti-rabbit PDGF antibody (Ferns, G. A. A., et al., 253 *Science,* 1129–1132 (1991), terbinafine (Nemecek, G. M. et al., 248 J. Pharmacol. Exp. Thera., 1167–11747 (1989), trapidil (Liu, M. W. et al., 81 *Circulation,* 1089–1093 (1990), interferon-gamma (Hansson, G. K. and Holm, 84 *J. Circulation,* 1266–1272 (1991), steroids (Colburn, M. D. et al., 15 J. Vasc. Surg., 510–518 (1992), see also Berk, B. C. et al., 17 J. Am. Coll. Cardiol., 111B–117B (1991), ionizing radiation (ref), fusion toxins (ref) antisense oligonucleotides (ref), gene vectors (ref), and rapamycin (see below).

Of particular interest in rapamycin. Rapamycin is a macrolide antibiotic which blocks IL-2-mediated T-cell proliferation and possesses antiinflammatory activity. While the precise mechanism of rapamycin is still under active investigation, rapamycin has been shown to prevent the G_1 to S phase progression of T-cells through the cell cycle by inhibiting specific cell cyclins and cyclin-dependent protein kinases (Siekierka, Immunol. Res. 13: 110–116, 1994). The antiproliferative action of rapamycin is not limited to T-cells; Marx et al. (Circ Res 76:412–417, 1995) have demonstrated that rapamycin prevents proliferation of both rat and human SMC in vitro while Poon et al. have shown the rat, porcine, and human SMC migrating can also be inhibited by rapamycin (J Clin Invest 98: 2277–2283, 1996). Thus, rapamycin is capable of inhibiting both the inflammatory response known to occur after arterial injury and stent implantation, as well as the SMC hyperproliferative response. In fact, the combined effects of rapamycin have been demonstrated to result in a diminished SMC hyperproliferative response in rat femoral artery graft model and in both rat and porcine arterial balloon injury models (Gregory et al., Transplantation 55:1409–1418, 1993; Gallo et al., in press, (1997)). These observations clearly support the potential use of rapamycin in the clinical setting of post-angioplasty restenosis.

Although the ideal agent for restenosis has not yet been identified, some desired properties are clear: inhibition of local thrombosis without the risk systemic bleeding complications and continuous and prevention of the dequale of arterial injury, including local inflammation and sustained prevention smooth muscle proliferation at the site of angioplasty without serious systemic complications. Inasmuch as stents prevent at least a portion of the restenosis process, an agent which prevents inflammation and the proliferation of SMC combined with a stent may provide the most efficacious treatment for post-angioplasty restenosis.

Experiments

Agents: Rapamycin (sirolimus) structural analogs (macrocyclic lactones) and inhibitors of cell-cycle progression.

Delivery Methods:

These can vary:

Local delivery of such agents (rapamycin) from the struts of a stent, from a stent graft, grafts, stent cover or sheath.

Involving comixture with polymers (both degradable and nondegrading) to hold the drug to the stent or graft.

or entrapping the drug into the metal of the stent or graft body which has been modified to contain micropores or channels, as will be explained further herein.

or including covalent binding of the drug to the stent via solution chemistry techniques (such as via the Carmeda process) or dry chemistry techniques (e.g. vapour deposition methods such as rf-plasma polymerization) and combinations thereof.

Catheter delivery intravascularly from a tandem balloon or a porous balloon for intramural uptake

Extravascular delivery by the pericardial route

Extravascular delivery by the advential application of sustained release formulations.

Uses: for inhibition of cell proliferation to prevent neointimal proliferation and restenosis.

prevention of tumor expansion from stents

prevent ingrowth of tissue into catheters and shunts inducing their failure.

1. Experimental Stent Delivery Method—Delivery from Polymer Matrix

Solution of Rapamycin, prepared in a solvent miscible with polymer carrier solution, is mixed with solution of polymer at final concentration range 0.001 weight % to 30 weight % of drug. Polymers are biocompatible (i.e., not elicit any negative tissue reaction or promote mural thrombus formation) and degradable, such as lactone-based polyesters or copolyesters, e.g., polylactide, polycaprolactonglycolide,polyorthoesters, polyanhydrides; polyaminoacids; polysaccharides; polyphosphazenes; poly (ether-ester) copolymers, e.g., PEO-PLLA, or blends thereof. Nonabsorbable biocompatible polymers are also suitable candidates. Polymers such as polydimethylsiolxane; poly(ethylene-vingylacetate); acrylate based polymers or copolymers, e.g., poly(hydroxyethyl methylmethacrylate, polyvinyl pyrrolidinone; fluorinated polymers such as polytetrafluoroethylene; cellulose esters.

Polymer/drug mixture is applied to the surfaces of the stent by either dip-coating, or spray coating, or brush coating or dip/spin coating or combinations thereof, and the solvent allowed to evaporate to leave a film with entrapped rapamycin.

2. Experimental Stent Delivery Method—Delivery from Microporous Depots in Stent Through a Polymer Membrane Coating:

Stent, whose body has been modified to contain micropores or channels is dipped into a solution of Rapamycin, range 0.001 wt % to saturated, in organic solvent such as acetone or methylene chloride, for sufficient time to allow solution to permeate into the pores. (The dipping solution can also be compressed to improve the loading efficiency.) After solvent has been allowed to evaporate, the stent is dipped briefly in fresh solvent to remove excess surface bound drug. A solution of polymer, chosen from any identified in the first experimental method, is applied to the stent as detailed above. This outerlayer of polymer will act as diffusion-controller for release of drug.

3. Experimental Stent Delivery Method—Delivery Via Lysis of a Covalent Drug Tether

Rapamycin is modified to contain a hydrolytically or enzymatically labile covalent bond for attaching to the surface of the stent which itself has been chemically derivatized to allow covalent immobilization. Covalent bonds such as ester, amides or anhydrides may be suitable for this.

4. Experimental Method—Pericardial Delivery

A: Polymeric Sheet Rapamycin is combined at concentration range previously highlighted, with a degradable polymer such as poly(caprolactone-gylcolide) or non-degradable polymer, e.g., polydimethylsiloxane, and mixture cast as a thin sheet, thickness range 10μ to 1000μ. The resulting sheet can be wrapped perivascularly on the target vessel. Preference would be for the absorbable polymer.

B: Conformal Coating: Rapamycin is combined with a polymer that has a melting temperature just above 37° C., range 40°–45° C. Mixture is applied in a molten state to the external side of the target vessel. Upon cooling to body temperature the mixture solidifies conformally to the vessel wall. Both non-degradable and absorbable biocompatible polymers are suitable.

As seen in the figures it is also possible to modify currently manufactured stents in order to adequately provide the drug dosages such as rapamycin. As seen in FIGS. 1*a*, 2*a* and 3*a*, any stent strut **10**, **20**, **30** can be modified to have a certain reservoir or channel **11**, **21**, **31**. Each of these reservoirs can be open or closed as desired. These reservoirs can hold the drug to be delivered. FIG. **4** shows a stent **40** with a reservoir **45** created at the apex of a flexible strut. Of course, this reservoir **45** is intended to be useful to deliver rapamycin or any other drug at a specific point of flexibility of the stent. Accordingly, this concept can be useful for "second generation" type stents.

In any of the foregoing devices, however, it is useful to have the drug dosage applied with enough specificity and enough concentration to provide an effective dosage in the lesion area. In this regard, the reservoir size in the stent struts must be kept at a size of about 0.0005" to about 0.003". Then, it should be possible to adequately apply the drug dosage at the desired location and in the desired amount.

These and other concepts will are disclosed herein. It would be apparent to the reader that modifications are possible to the stent or the drug dosage applied. In any event, however, the any obvious modifications should be perceived to fall within the scope of the invention which is to be realized from the attached claims and their equivalents.

What is claimed is:

1. A stent having a coating containing rapamycin, said coating formed from a polymer mixed carrier containing the rapamycin; and said coating applied to said stent.

2. The stent of claim **1** wherein the stent is dip-coated.

3. The stent of claim **1** wherein the stent is sprayed with said coating.

4. A stent having a coating containing rapamycin or its analogs, wherein said rapamycin or said analogs are contained in the coating at a weight percentage of 0.0001% to 30%.

5. The stent of claim **4** wherein a polymer is mixed to the rapamycin or its analogs.

6. The stent of claim **4** wherein a polymer is bound to the rapamycin or its analogs.

7. The stent of claim **4** wherein the rapamycin or its analogs is entrapped on the surface of the stent by a polymer.

8. A stent having a coating containing rapamycin, said coating formed from a polymer mixed carrier containing the rapamycin or its analogs; and said coating applied to said stent; wherein the polymer is biocompatible and degradable; and

wherein the polymer is chosen from: lactone-based polyesters, lactone-based copolyesters; polyanhydrides; polyaminoacids; polysaccharides; polyphosphazenes; poly(ether-ester) copolymers, and blends of such polymers.

9. A stent having a coating containing rapamycin, said coating formed from a polymer mixed carrier containing the rapamycin or its analogs; and said coating applied to said stent; and

wherein the polymer is chosen from: lactone-based polyesters, lactone-based copolyesters; polyanhydrides; polyaminoacids; polysaccharides; polyphosphazenes; poly(ether-ester) copolymers, and blends of such polymers.

10. A stent having a coating containing rapamycin, said coating formed from a polymer mixed carrier containing the rapamycin or its analogs; and said coating applied to said stent; wherein the polymer is nonabsorbable; and

wherein the polymer is chosen from: polydimethylsiolxane; poly(ethylene)vinylacetate; poly(hydroxy) ethylmethylmethacrylate, polyvinyl pyrrolidone; polytetrafluoroethylene; and cellulose esters.

11. A stent having a coating containing rapamycin or its analogs, said coating formed from a polymer mixed carrier containing the rapamycin or its analogs; and said coating applied to said stent; and

wherein the polymer is chosen from: polydimethylsiolxane; poly(ethylene)vinylacetate; poly(hydroxy) ethylmethylmethacrylate, polyvinyl pyrrolidone; polytetrafluoroethylene; and cellulose esters.

12. A stent having a coating containing rapamycin or its analogs, said coating formed from a polymer mixed carrier containing the rapamycin or its analogs; and said coating applied to said stent; and further comprising:

a generally thin walled cylinder, said cylinder containing a plurality of generally solid struts, said applied to said strut, and a channel formed in at least one of said struts, said channel having a closed perimeter on all sides and an open top, and said channel smaller in all dimensions than said strut, said channel containing a reservoir of said rapamycin coating applied therein.

13. A stent having a coating containing rapamycin or its analogs, wherein said rapamycin or said analogs are contained in the coating at a weight percentage of 0.0001% to 30%, wherein the coating is a polymer.

14. The stent of claim **13** wherein said polymer is mixed to the rapamycin or its analogs.

15. The stent of claim **4** wherein said polymer is bound to the rapamycin or its analogs.

16. The stent of claim **13** wherein the rapamycin or its analogs is entrapped on the surface of the stent by said polymer.

17. A stent containing a polymer and rapamycin or its analogs wherein said rapamycin or its analogs are contained in a therapeutically beneficial amount to combat restenosis.

18. The stent of claim **17** wherein said polymer is mixed to the rapamycin or its analogs.

19. The stent of claim **17** wherein said polymer is bound to the rapamycin or its analogs.

20. The stent of claim **17** wherein the rapamycin or its analogs is entrapped on the surface of the stent by said polymer.

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